Spinal cord transection and intrathecal injection of BDNF : two relevant models of neuropathic pain in rats ?

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Transection spinale et injection intrathécale de BDNF : Deux modèles pertinents de douleur neuropathique chez le rat ?

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Les douleurs neuropathiques, celles qui sont provoquées par des lésions du système nerveux central ou périphérique, sont les plus difficiles à traiter du fait de leur résistance aux traitements antalgiques classiques. Les traitements utilisés aujourd'hui font appel à des classes thérapeutiques non spécifiquement ciblées sur la douleur, en particulier des antidépresseurs et des anticonvulsivants. Leur efficacité limitée ne repose en fait que sur des observations empiriques. Une meilleure connaissance des processus physiopathologiques sous-tendant les douleurs neuropathiques constitue un préalable à toute innovation thérapeutique, et c'est à cette fin que je me suis appliqué à développer deux modèles de douleurs neuropathiques chez le rat pour en étudier les caractéristiques comportementales, fonctionnelles, cellulaires et biochimiques. Le premier modèle visait à l'induction d'une douleur neuropathique *centrale* provoquée par la section complète de la moelle épinière au niveau thoracique (T8-T9) ; le second a consisté à injecter, directement au niveau spinal, par voie intrathécale (i.t.), le facteur neurotrophique BDNF (Brain Derived Neurotrophic Factor ; dont l'implication dans les voies de signalisation nociceptive est bien établie dans la littérature). Dans les deux cas, les conséquences pro-algiques de ces interventions ont été comparées à celles induites par la ligature unilatérale du nerf sciatique, qui constitue encore aujourd'hui un modèle classique, mais très imparfait, d'une douleur neuropathique *périphérique*.

Dès le 2ème jour après la section spinale, et jusqu'au moins deux mois plus tard, les rats lésés présentent une forte allodynie mécanique (test des filaments de von Frey) dans le territoire cutané juste en avant de la lésion. Cet effet traduit bien une neuropathie centrale car il n'existe pas chez les rats « sham » qui ont subi l'intégralité de l'intervention chirurgicale à l'exception de la section spinale. L'allodynie mécanique est associée à une induction significative de l'expression (RTqPCR) de marqueurs de souffrance neuronale (ATF-3) et d'activation microgliale (OX-42, récepteurs P2X4, P2X7 et TLR4) et astrocytaire (GFAP), ainsi que du BDNF et de cytokines pro-inflammatoires (IL-1ß, IL-6, TNF-α), mais de façon plus transitoire, ceci dans les ganglions de racines dorsales et/ou la moelle épinière dorsale (comme à la suite de la ligature du nerf sciatique, mais avec des cinétiques différentes). Pour sa part, l'injection intrathécale i.t. d'une dose infra-nanomolaire unique de BDNF (0.3 – 3.0 ng) induit aussi une forte allodynie et une hyperalgésie mécaniques, au niveau des pattes postérieures, qui se développent en 3-5 jours, et perdurent pendant deux semaines. Cependant, au contraire de la section spinale (et de la ligature du nerf sciatique), l'injection i.t. de BDNF ne provoque pas d'activation microgliale ni d'induction de cytokines. Elle entraine en revanche une auto-induction du BDNF, qui semble clé pour l'hyperalgésie puisque celle-ci peut être, en grande partie, supprimée par l'administration d'un inhibiteur du récepteur TrkB du BDNF, la cyclotraxine B (20 mg/kg i.p.), comme d'ailleurs l'allodynie induite par la ligature du nerf sciatique.

Au plan pharmacologique, un antalgique opiacé comme le tapentadol s'est révélé efficace dans les deux modèles**.** De même, les anticonvulsivants, comme la prégabaline et la gabapentine, ont réduit la douleur neuropathique chez les rats injectés par le BDNF i.t. et chez les rats CCI-SN.

En conclusion, il semble que l'injection intrathécale de BDNF, qui évite la réalisation de lésions par intervention chirurgicale, puisse constituer un nouveau modèle pertinent de douleur neuropathique chez le rat. De plus, nos résultats laissent à penser que le blocage de la voie de signalisation BDNF-TrkB pourrait ouvrir de nouvelles pistes pour la réduction des douleurs

neuropathiques *périphériques*. Il reste à explorer si cette piste serait aussi pertinente dans le cas de la douleur neuropathique *centrale* consécutive à une lésion spinale.

SOMMAIRE

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ABREVIATIONS

5-HT : 5-Hydroxytryptamine ou sérotonine

8-OH-DPAT : 8-Hydroxy-2-(di-n-propylamino)-tétraline

AC : Adénylate cyclase

ADN : Acide désoxyribonucléique

AMPA : Acide α-amino-3-hydroxy-5-méthyl-4-isoxazole propionique

AMPc : Adénosine monophosphate cyclique

ARN : Acide ribonucléique

ASIA : American Spinal Injury Association

ARNm : ARN messager

ASIC : Acid-sensing ion channel

ATF3 : Activating transcription factor 3

ATP : Adénosine triphosphate

BDNF : Brain-derived neurotrophic factor

CaMKII : Ca2+/calmodulin-dependent protein kinase II

CCK : Cholecystokinin

CCI : Chronic constriction injury

CGRP : Calcitonin-gene related peptide

CHIP : chromatin immunoprecipitation

COX : Cyclooxygénase

EPSC : excitatory post synaptic current

ERK : Extracellular signal-regulated kinase

GABA : Acide gamma-aminobutyrique

GFAP : Glial fibrillary acidic protein

GRD : Ganglion de racine dorsale

HIV : Human immunodeficiency virus

HSV-1 : Herpes simplex virus

I.A.S.P. : International Association for the Study of Pain

IPSC : Inhibitory post synaptic current

i.p. : Voie intrapéritonéale

i.v. : Voie intraveineuse

i.t. : Voie intrathécale

IL : Interleukine

JNK : c-Jun N-terminal Kinase

KCC2 : K+/Cl- co-transporter 2

KO : Knock out

Kv : Canaux potassium voltage-dépendants

LRt : Noyau réticulé latéral

LPS : lypopolysaccharides

LTP : Long term potentiation

MAPK : Mitogen-activated protein kinase

MCP-1 : monocyte chimioattractant protein 1

MEK : ou MAP2K, Mitogen-activated protein kinase kinase

NA : Noradrénaline

Nav : Canaux sodium voltage-dépendants

NGF : Nerve growth factor

NKCC : Na^+ -K^+ -Cl^- exporter channels

NMDA : N-méthyl-D-aspartate

NO : Monoxyde d'azote (oxyde nitrique)

NT3 : Neurotrophin 3

NT4/5 : Neurotrophins 4/5

OX42 : Anticorps des récepteurs de type 3 du complément (marqueur d'activation microgliale)

P2X : Récepteurs purinergiques ionotropiques

p38-MAPK : p38-mitogen-activated protein kinase

PA : Potentiel d'action

PBil : Partie interne latérale de l'aire parabrachiale

PBl : Aire parabrachiale latérale

PI3K : Phosphatidylinositol 3-kinase

PKA : Protéine kinase A

PKC : Protéine kinase C

PLC : Phospholipase C

Po : Complexe postérieur du thalamus latéral

POH : Région préoptique de l'hypothalamus

PVH : Noyau paraventriculaire de l'hypothalamus

qRT-PCR : Quantitative reverse transcription - polymerase chain reaction

QVL : Quadrant antéro-latéral de la moelle épinière

s.c. : Voie sous-cutanée

SCI : Spinal cord injury

SCT : spinal cord transection

SGPA : Substance grise périaqueducale

SP : Substance P

Sp5c : Sous-noyau caudalis du noyau spinal du trijumeau

Sp5i : Sous-noyau interpolaris du noyau spinal du trijumeau

Sp5o : Sous-noyau oralis du noyau spinal du trijumeau

SRD : Subnucleus reticularis dorsalis

STZ : Streptozotocin

TLR : Toll-like receptor

TNFα : Tumor necrosis factor alpha

Trk : Tropomyosin-receptor kinase

TRP : récepteur ionotropique de type « transient receptor potential »

TTX : Tétrodotoxine

VMH : Noyau ventromédian de l'hypothalamus

VMl : Noyau ventromédial latéral du thalamus médian

VPI : Noyau ventropostéro-inférieur du thalamus latéral

VPL : Noyau ventropostéro-latéral du thalamus latéral

VPM : Noyau ventropostéro-médian du thalamus latéral

ZPP : Zone of partial preservation

RAPPELS BIBLIOGRAPHIQUES

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CHAPTER I : INTRODUCTION ON PAIN

I.1. Pain definition

Pain is difficult to define. However it is essential for the clinicians to discriminate between the different pain conditions in order to provide the most appropriate treatment to relieve patients of severe pain. The International Association for the Study of Pain (I.A.S.P.) has been founded in 1973 with such goals. Nowadays, I.A.S.P. is the leading professional forum for science, practice and education in the field of pain. Acknowledged by the World Health Organization as a non-governmental organization in 1987, I.A.S.P. has now more than 7,900 members in 133 countries. I.A.S.P. defines pain in those terms: "*Pain is an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage*" (Merskey ,1994). This definition insists on the pluridimensional aspects of pain. Pain arises from generator mechanisms, which induce a psycho-physical experience implying cognitive processes and triggering motor, verbal and physiological responses. Therefore, I.A.S.P. definition covers very complex integrative mechanisms which are initiated in lesioned tissues/nervous system and end with activation of sub-cortical and cortical areas where pain sensation is generated. In a simplified way, pain can be resumed into 4 principal aspects which all contribute to the painful sensation*: sensori-discriminative, emotional and affective, cognitive* and *behavioral* (Melzack and Casey, 1968).

I.2. The different aspects of pain

The *sensori-discriminative* aspect of pain is encoded by neurophysiological mechanisms of nociception. These mechanisms allow assessment by the subject of the quality (burn, electric-shock, torsion), the duration and evolution (brief, persistent, chronic, recurrent), the intensity and the localization of pain sensation evoked by stimuli likely to provoke tissue lesion.
The *emotional and affective* aspect of pain is defined by the aversive, unpleasant, and tough feelings attached with pain. This aspect can lead to mental disorders such as anxiety and depression (Boureau et al., 1997). It is conditioned not only by the stimulus itself, but also by the context in which it occurs. Among other factors, uncertainty on the evolution of underlying disease can markedly influence the affective aspect of pain.
The whole mental processes that lead to understanding the reasons for pain occurrence, to assessing its psycho-sensory characteristics and allow behavioral adaptive responses represent

the *cognitive* aspect of pain. It includes attention, anticipation and diversion processes as much as the interpretation and the value attributed to pain, reference to previous pain experiences, semantic expression of pain sensation, which will all be determinant for the patient to choose the most appropriate behavior to adopt (Beecher, 1959).
The *behavioral* aspects are all the verbal and non-verbal manifestations of a suffering person (complaints, facial expressions, antalgic positions, inability to stand normally), with associated vegetative responses and reflexes (Fordyce ,1978). Those manifestations are reactions proportional to the pain feeling but are also a way to communicate with surrounding persons.

I.3. Different types of pain

Brief pain

It results from nociceptors activation without tissue lesion. It is a way for the organism to protect its integrity and its survival from more painful harm. Even though only humans actually express the painful perception of a brief pain, it is highly probable that this type of pain exists for any organism with a nervous system.

Acute pain

Acute pain is triggered by nociceptors activation during tissue lesion. Like brief pain, its role is to protect the organism from further harmful stimulations. Despite tissue inflammation resulting from the lesion, repair mechanisms are efficient, which means that no medical assistance is necessary in theory, and pain has only a protective role of the injured tissue. However, a medical intervention is recommended to prevent from other wounds, to reduce the painful sensation and to accelerate healing. Post traumatic and surgical interventions are considered as acute pain.

Chronic pain

It arises from important tissue lesion or pathologies. In that case, homeostatic dysregulations are strong enough to disrupt repair systems, which can no longer heal the pathology causes. Indeed, a nerve lesion can, by itself, induce a vicious circle: healing mechanisms will prevent the organism to return to its normal state of nociceptive transmission. This will induce a persistent release of factors that are responsible for pain, when the initial cause of pain is no longer present. It is generally agreed that three different types of chronic pain can be defined: pain induced by overstimulation, neurogenic pain and psychogenic pain.

Pain induced by overstimulation

Overstimulation of the nociceptive pathways is the major reason for acute pain. It becomes chronic in persistent lesion pathologies refractory to treatments and where healing repair mechanisms are inefficient. Nociceptive pathways are thus continuously activated because of the permanent presence of triggering-pathology elements. Persistent rheumatoid pain and pain associated with some cancers have the characteristics of this kind of pain. Their treatment consists of healing the pathological state causing the excessive activation of nociceptive pathways or blocking the neural transmission of nociceptive signaling with central or peripheral analgesics.

Neurogenic pain

Neurogenic pain is most often called painful neuropathy or neuropathic pain. In that case, abnormal painful sensation is generated by lesion of the central or peripheral somatosensory nervous system.

Generally, pain arises in the territory innervated by the lesioned nerve and is sometimes associated with an important lack of thermal and mechanical sensitivity. Therefore, nerve lesion can lead to paradoxical situations where the patient can suffer from both positive symptoms (abnormal feelings, pain) and sensory deficits.

Two types of mechanisms can be responsible for neurogenic pain: a neural tissue compression (affecting a peripheral nerve, a root or a plexus) or a nerve lesion secondary to various diseases (diabetes and other metabolic disorders, viral infections, cancers, etc). Neuropathic pain can be symptomatic of polyneuropathies, which affect nervous pathways in a diffuse and symmetrical way, and mononeuropathies (Bouhassira and Attal, 1997). The latter are considered “simple” when they concern only a nerve trunk or “multiple” when they touch numerous trunks, roots or even plexuses but in an asymmetric way.

Psychogenic pain

Psychogenic pain defines a pain really felt by the patient but without apparent lesion. Physiopathological mechanisms underlying this type of pain are essentially unknown, which leads to qualify psychogenic pain as idiopathic pain (Stoudemire and Sandhu, 1987). Moreover, psychogenic pain is mostly refractory to antalgics. Clinically, psychogenic pain includes pathologies with symptoms corresponding either to a precise semiological framework such as

tension headache, fibromyalgia, glossodynia… or to variable description using terms of poor clinical significance.

As a matter of fact, psychogenic pain is not only characterized by the absence of clinically detectable lesion. In addition, it has to be associated with psychopathological symptoms such as depression, hypochondria, hysteric conversion or somatization of emotional disorders.

Actually, chronic pain has only rarely a "pure" psychogenic origin. In most cases, it is underlain by both somatic and psychosocial suffering. A patient's description only in physical or psychological terms does not take into account the fact that pain has both physiopathological and affective components, as judiciously emphasized in the I.A.S.P.'s definition (Merskey, 1994).

CHAPTER II: PHYSIOLOGY OF NOCICEPTION

II.1. Nociceptors

The somato-sensory system comprises specific receptors that discriminate between the different modalities of stimulation (mechanical, thermal, nociceptive…) affecting a subject, for the central nervous system to process the information, identify any potential threat and produce an adapted response.

Nociceptive (and thermal non nociceptive) information is perceived by a unique type of nerve endings within amyelinic arborizations. Activation of membrane receptors located on those nerve endings is the first step toward sensory message integration. Those receptors are not all well identified and their respective roles are not clearly established, but genetically modified mice (especially knock out mutants) allowed the clear-cut demonstration of the implication of transient receptor potential channels (TRP) in sensitivity to thermal (nociceptive or not) and - at least - some chemical stimulations (Woolf and Ma, 2007; Basbaum et al., 2009) and acid-sensing ion channels (ASIC) in pH and mechanical sensitivity.

Heat stimulation is perceived through TRPV1 receptors which are activated by temperatures $\geq 43°C$ and by capsaicin, and TRPV2 receptors, which temperature threshold is $\geq 50°C$. For non-nociceptive heat temperatures, TRPV3 and TRPV4, with activation thresholds of 25°C and 35°C, respectively, are implicated (Caterina et al., 1997; Basbaum et al., 2009).
On the other hand, the TRPM8 receptor, which is activated by temperatures $\leq 28°C$ and by menthol, seems to play a key role in the perception of non-nociceptive cold stimulations (Reid and Flonta, 2001), and the TRPA1 receptor allows probably non-specific perception of cold, mechanical and chemical stimulations. Indeed, it is activated by menthol, and an ortholog of this receptor in *C.elegans* is implicated in the perception of mechanical stimulations (Hinman et al., 2006; MacPherson et al., 2007). However, since TRPA1 can be activated by different irritating molecules such as isothiocyanate, thiosulfinate and acroleine (respective components of mustard, garlic, and tear gas), its principal function would be the perception of chemical stimulations. Along with these observations, knock-out mice deficient in TRPA1 (TRPA1-/-) display a marked reduction in sensitivity to these molecules (Caceres et al., 2009). A summary of the main characteristics and functional implications of TRP channels is illustrated in Figure 1.

On the other hand, sensitivity to extracellular acidification generated by pain-inducing tissue lesions and to some nociceptive mechanical stimulations is encoded by ASICs. ASIC 1 and ASIC 3 channels are activated by moderate acidification corresponding to a pH_{50} of 6.6-6.8, whereas ASIC 2a currents are triggered by stronger acidification corresponding to a pH_{50} of 4.9. ASIC 1a and ASIC 2 channels have also a role in mechanical nociception. Activation of ASIC 1a leads to mechanical hyperalgesia (Duan et al., 2012) and ASIC 2 seems to be necessary for mechanical transduction (McIlwrath et al., 2005). Table 1 summarizes the principal characteristics of ASIC subtypes.

Amyelinic nerve endings involved in nociceptive (and thermal non nociceptive) sensory function are the terminals of Aδ and C fibers. Depending on the nature of the molecular receptors present on nerve endings, those fibers can convey different kinds of nociceptive messages. Aδ and C fibers markedly differ from large diameter (5-20 μm) Aα/β myelinated fibers with high speed conduction of action potentials (35-120 $m.s^{-1}$) which, under physiological conditions, convey tactile and proprioceptive informations but no nociceptive messages.

II.2. Primary afferent fibers

II.2.a. Aδ fibers

Aδ fibers are faintly myelinated and have a medium size diameter (1-5 μm). Those fibers have an intermediate velocity conduction of action potentials (4-30 $m.s^{-1}$) and convey thermal information (for non-nociceptive temperatures between 10°C and 42°C) as well as nociceptive messages whose characteristics depend on the specificity of their receptors.
There are indeed two types of Aδ nociceptors. The first ones are activated by high temperatures and the other ones are sensitized by tissue lesion, but both respond to high intensity mechanical stimulations. Moreover, some Aδ nociceptors are also activated by moderate cold stimulations. Aδ fibers convey a fast nociceptive feeling, sting-like, brief, localized and intense which provokes a reflex response aimed at removing the body from the injuring source (Julius and Basbaum, 2001). Lesions of Aδ fibers can lead to pathological nociceptive perception of non-nociceptive stimuli (Millan, 1999; Woolf, 2004).

II.2.b. C fibers

C fibers represent 60 to 90% of the whole pool of cutaneous nerve fibers. They are amyelinic, have a small size diameter (≤1.5 μm) and slowly convey action potentials (0.5-2 $m.s^{-1}$). Most of

C-fiber nociceptors are considered as polymodal because they can encode diverse types of nociceptive stimulations (mechanical, thermal and chemical). In contrast to Aδ fibers which are implicated in the first brief and localized pain sensation, C fibers activation induces a more delayed painful sensation, burning-like, diffuse and persistent (Julius and Basbaum, 2001). Twenty per cent of polymodal nociceptors are thought to be in a « silence » state under physiological conditions, but can be activated under pathological conditions, notably in inflamed tissues (Schmidt et al., 1995)

II.3. Nociceptive pathways

II.3.a. Spinal cord laminae innervated by primary afferent fibers

Primary sensory fibers of Aα/β, Aδ and C types come from neurons contained in dorsal root ganglia (DRG) located on each side of the spinal cord. They convey sensory information from where the periphery to the spinal cord where they establish synaptic contacts with second-order neurons. After bypassing lamina I, Aβ fibers are divided into two contingents : the first one contacts principally neurons in laminae III and IV and, to a lesser extent, laminae II and V neurons (Rexed, 1952; Besson and Chaouch, 1987), whereas the second one constitutes the dorsal lemniscal column ascending to the nucleus gracilis and the nucleus cuneatus within the medulla oblongata. On the other hand, Aδ and C fibers project essentially in the dorsal horn superfical laminae I and II (Rexed, 1952), but reach also deep laminae V, VI, VII and X (Bernard and Villanueva, 2009) (see Figure 2).

II.3.b. Second order neurons.

There are two types of the so-called second order neurons which receive inputs from primary afferent fibers, and project into supraspinal centers:

- *Nociceptive specific neurons*, which respond only to intense nociceptive stimulations. They are mostly localized in lamina I and in the outer lamina II. Nevertheless, some nociceptive specific neurons can also be found in lamina V, but to a lesser extent.

- *Convergent nociceptive neurons*, also called non-specific nociceptive neurons, are mostly localized in lamina V and at a lower density in dorsal horn superficial laminae (Dubner and Hargreaves, 1989). These neurons respond to various types of broad-range intensity stimuli, nociceptive or not. Through their localization, convergent nociceptive neurons can establish contacts with all kinds of fibers and thus are the first step of pain message integration.

II.3.c. Ascending spinal-supraspinal nociceptive pathways

Second order neurons project into cerebral structures. Most of their axons form a column of ascending fibers in the contra-antero-lateral quadrant of the white matter of the spinal cord. A small contingent of axons, particularly those of neurons in lamina V, does not cross the median line and projects directly into ipsilateral supraspinal structures. Their role in the physiological integration of nociceptive information is still a matter of debate.

Functional anatomy studies identified three main pathways for the transfer of nociceptive signals from dorsal horn spinal cord neurons to the cerebral cortex where pain sensation is generated (Gauriau and Bernard, 2002).

II.3.c.1. The spino-thalamic tract (Fig. 3)

Nociceptive specific neurons projections from superficial laminae are divided into two main pathways. The first one, called the spino-thalamic tract, projects into the ventro-posterolateral nucleus of the thalamus (Craig, 1991). This pathway ends in the cortex, notably in somatosensory areas S1 and S2. The sensori-discriminative aspect of pain is integrated in the ventro-posterolateral nucleus which encodes the duration, the intensity and the localization of nociceptive stimulation.

II.3.c.2. The spino-parabrachial tract (Fig. 3)

The second pathway issued from lamina I nociceptive neurons projects into the latero-external parabrachial area (Bernard and Besson, 1990). Messages relayed in parabrachial area are conveyed mainly into the amygdala and the ventro-median nucleus of the hypothalamus (Bernard and Besson, 1990; Bester et al., 1995), and to a lesser extent into the periaqueductal grey (Bandler and Shipley, 1994; Craig, 1995). The amygdala seems to play an important role in the anxiogenic aspect of pain and in fear-conditioned learning (Manning et al., 2001), whereas the hypothalamus and the periaqueductal grey participate in humoral and vegetative responses triggered by nociceptive stimulations (Chamberlin and Saper, 1992; Malick et al., 2001).

Figure 3: Lamina I (LI) nociceptive pathways. Axons from the spinal cord - dorsal horn lamina I neurons cross the median line at their segmental origin. They gather into the antero-lateral quadrant (QVL) before ascending to the medulla. These neurons project essentially into the lateral parabrachial area (PBI), the periaqueductal grey (SGPA) and the lateral thalamus. The lateral parabrachial area then projects into the amygdala and the hypothalamus. Neurons in lateral thalamus nuclei project into the primary (S1) and secondary (S2) somatosensory and insula cortex, as well as in the amygdala. Line thickness reflects the density of the tracts conveying nociceptive messages (from Bernard and Villanueva, 2009).
LH: lateral hypothalamus; LI: dorsal horn and Sp5c lamina I; Po: lateral thalamus posterior complex; POH: preoptic hypothalamic nucleus ; PVH: paraventricular hypothalamic nucleus;VMH : ventromedian hypothalamic nucleus;VPI: ventro-postero-inferior lateral thalamic nucleus;VPL ventro-postero-lateral thalamic nucleus; VPM: ventro-postero-median thalamic nucleus.

II.3.c.3. The spino-reticular tract (Fig. 4)

Convergent non-specific nociceptive neurons from deep laminae form the spino-reticular tract. Activation of this pathway triggers the somatic motor response and participates in the emotional aspect of pain.

The motor response to nociceptive stimulation results from neuronal activation in the lateral reticular nucleus, the dorsal reticular subnucleus, and the gigantocellular reticular nucleus in the medulla which ends with the activation of motoneurons within the ventral horn of the spinal cord and the trunk motor nucleus (Bernard and Besson, 1990; Villanueva and Le Bars, 1995).
Emotional response involves the gigantocellular reticular nucleus, the dorsal reticular subnucleus and the internal lateral parabrachial subnucleus which project into different thalamus subnuclei (Bester et al., 1995; Villanueva et al., 1998) before reaching the striatum and the cingular and prefrontal cortical areas (Berendse and Groenewegen, 1991; Desbois and Villanueva, 2001).

CHAPTER III: CHRONIC NEUROPATHIC PAIN

Pain is considered as chronic when it persists, with or without treatment, for more than 6 months (Bonica, 1990). Among the different types of chronic pain, neuropathic pain is caused by lesion or dysfunction of central or peripheral nervous system. It can be provoked by nerve compression or section, spinal cord contusion, viral infection (herpes zoster, varicella zoster), chemicals (notably anti-cancer and anti-retrovirus drugs) or metabolic pathology such as diabetes mellitus. In addition, neuropathic pain constitutes one of the most deleterious symptoms of several neurological syndromes (multiple sclerosis, amyotrophic lateral sclerosis, Parkinson's disease, Guillan-Barré syndrome, stroke).

III.1- Sensory dysfunctions associated with neuropathies

Neuropathies are characterized by phenotypical modifications affecting lesioned nerve fibers, which underlie non-painful and painful sensory dysregulations.

III.1.a. Non-painful sensory dysregulations

Two different types of sensations enter into this category: on the one hand, paresthesias, which correspond to abnormal sensations like tingling or numbness, and, on the other hand, dysesthesias, which are similar sensations but with additional unpleasant feeling. Respective diagnosis as well as identification of underlying physiopathological mechanisms are difficult because the distinction between these two types of sensory dysregulation depends entirely on patient's feelings.

III.1.b. Painful dysregulations

Like non-painful sensory dysregulations, painful sensations can be evoked or spontaneous. Most of the time, spontaneous pain is described as persistent and diffuse burning-like sensation with no specific location in given organs or tissues. In addition, patients suffer from paroxystic pain, which is described as electric shock-like sensations of short duration alternating with remission periods.

Evoked painful sensations vary with the intensity of the stimulus (mechanical, thermal or chemical). Two types of sensations are reported by patients: hyperalgesia, which corresponds to exacerbated pain from a stimulus that normally induces only moderate pain, and allodynia,

which is pain caused by a stimulus that does not normally evoke pain in healthy subjects (Merskey, 1994). Allodynia is particularly debilitating because patients have to change their way of living and their social behavior, by avoiding every contact (with clothes, sheets, shower) that could eventually trigger pain in the body territory concerned. Actually, two types of allodynia, static and dynamic, have been described (Koltzenburg et al., 1992; Ochoa and Yarnitsky, 1993).

III.2. Chronic pain in spinal cord injury (SCI) patients.

In addition to motor and genito-urinary deficits, pain resulting from spinal cord injury (SCI) is an especially debilitating issue. The prevalence of severe chronic pain in SCI patients ranges from 30 to 51% (Donovan et al., 1982; Finnerup et al., 2001; Bryce et al., 2006, 2012). Their pain can be so severe that, when asked, SCI patients would rather renounce their sexual or bladder function than still feeling pain (Nepomuceno et al., 1979; Baastrup and Finnerup, 2008). Indeed, it affects patients quality of life much more than motor dysfunction and often leads to depression and even suicide (Donovan et al., 1982; Cairns et al., 1996; Rintala et al., 1998; Westgren and Levi, 1998; Widerström-Noga et al., 2001; Putzke et al., 2002; Attal et al., 2008).
Pain arising from SCI is multiple in terms of physiopathology, symptoms as well as body localization. SCI pain can be referred to as nociceptive (mainly musculoskeletal or visceral pain) or neuropathic (either above-, at- or below- the level of the injury) pain. Musculoskeletal pain has the highest prevalence with 58% of SCI patients suffering from it. Neuropathic pain is also frequent with 12-42% of patients reporting at-level pain and 23-34% reporting below- level pain (Baastrup and Finnerup, 2008; Bryce et al., 2012)

III.2.a. Classification of spinal cord injuries

Spinal cord injuries may lead to different sensory and motor dysfunctions depending on the level and extent of the injury. To classify the different cases of spinal cord injury patients, Frankel et al. (1969) created a classification depending on the SCI extent and the resulting sensory deficits. This classification has been improved by the American Spinal Injury Association (ASIA) which published a further revision recently (Kirshblum et al., 2011). This latter version distinguishes two main categories of patients with complete (ASIA A) or incomplete (ASIA B, C or D) spinal cord injury. ASIA A refers to a patient who does not have any sensory or motor function in the sacral segment S4-S5. Since a complete lesion is rarely seen, the definition of the Zone of Partial Preservation (ZPP) has been proposed for a more appropriate classification of ASIA A patients.

Indeed, ZPP is strictly applicable to ASIA A patients and refers to the most caudal zone with the dermatomes and/or myotomes still innervated, which preserve some sensory and/or motor functions. The other types of injury, defined as incomplete, and are listed in the table 2.

III.2.b. The various types of pain in SCI patients

III.2.b.1. Nociceptive pain

- *Musculoskeletal pain*

Musculoskeletal pain arises from musculoskeletal structures (muscles, tendons, ligaments, joints, bones) located above-, at- or below- the level of spinal cord injury. Typically, musculoskeletal pain arises when the patient moves and includes pain resulting from joint arthritis, spinal fractures, muscle injury and/or muscle spasms (Cardenas et al., 2002). Musculoskeletal structures show tenderness on palpation and imaging reveals the skeletal pathology, which fits with the pain representation. This pain, described as "dull" or "aching", can generally be alleviated by anti-inflammatory and opioid medications, at least better than neuropathic pain (Donovan et al., 1982; Bryce et al., 2006, 2012). Pain located in musculoskeletal tissues without those characteristics is considered as neuropathic pain.

- *Visceral pain*

Visceral pain is generated in visceral structures and located in the thorax, abdomen or pelvis. Tenderness of visceral structures is evident on palpation of the abdomen and the pathology is further characterized by imaging, in consistence with the pain representation. It is temporally correlated with food intake or visceral functions/dysfunctions such as constipation. The pain is described as "cramping", "dull" or "tender", and is associated with nausea and sweating. Treatments with anti-spasmodic drugs, histamine H2 receptor antagonists and inhibitors of proton pump can produce significant alleviating effects, in addition to the use of "classical" antalgics (acetaminophen, opioids) which are effective against severe visceral pain. Pain located in the thorax, abdomen or pelvis without those characteristics is considered as neuropathic pain.

III.2.b.2. Neuropathic pain

Neuropathic pain is caused by lesion or disease of the somatosensory nervous system. In the case of spinal cord injury, neuropathic pain refers to a lesion or a disease of a nerve root or the spinal cord itself. Its onset can be almost immediately after the SCI or months later (Baastrup and Finnerup, 2008). The faster the pain arises, the more likely the patient is to have on going pain

for 3 to 5 years after the injury (Siddall et al., 2003). Neuropathic pain is mainly characterized by its severity, with typical symptoms such as allodynia (see above) which is an especially disabling feature because touch from clothes or taking a shower may cause intense pain, and even a gentle touch can be enough to trigger a burning sensation (Baastrup and Finnerup, 2008). In SCI patients, induced neuropathic pain is commonly occurring at-level and below the lesion.

- *At-level neuropathic pain*

Pain seems to develop earlier at-level than below injury level (Siddall et al., 2003). To be defined as at-level pain, it has to be perceived at least within the dermatome concerned by the lesion and/or in one dermatome above or three below (Bryce et al., 2012). Any pain experienced below three dermatomes should not be described as at-level neuropathic pain. Moreover, to be classified as at-level pain, neuropathic pain has to result from a lesion or disease affecting a nerve root and/or the spinal cord itself closely related to the location of injury-evoked somatosensory dysfunctions.

At-level SCI pain is characterized by sensory deficits along with allodynia and hyperalgesia. Words used to describe this pain are “hot-burning”, “tingling”, “pricking”, “pins and needles”, “sharp”, “shooting”, “squeezing”, “painful cold” and “electric shock-like” ” (Donovan et al., 1982; Defrin et al., 2001; Putzke et al., 2002; Bryce et al., 2006, 2012; Attal et al., 2008).

- *Below-level neuropathic pain*

To be classified as below-level neuropathic pain, pain has to be perceived beyond three dermatomes under the level of injury and may extend up to the at-level dermatome. Below-level pain can occur in patients with complete or incomplete injuries (Werhagen et al., 2004; Bryce et al., 2012).

Like at-level pain, below-level neuropathic pain is characterized by sensory deficits along with allodynia and hyperalgesia within the affected dermatomes. In addition, the same words as those used to describe at-level pain are also privileged by below-level suffering patients to depict their painful symptoms (Donovan et al., 1982; Defrin et al., 2001; Putzke et al., 2002; Bryce et al., 2006, 2012; Attal et al.,2008).

CHAPTER IV: MODELISATION OF CHRONIC PAIN IN RODENTS

IV.1. Drugs and virus-induced neuropathic pain

IV.1.a. Diabetes-inducing drugs

Diabetes-induced neuropathy concerns about 8-16% of patients with diabetes mellitus (Dyck et al., 1993; Daousi et al., 2004; Wu et al., 2007) and comes as a late complication of these metabolic pathologies.

Patients develop abnormal sensations such as paresthesia, allodynia and hyperalgesia, but also spontaneous pain that coexists with loss of normal sensory function (Calcutt, 2002). The pathological features of diabetic neuropathy include consequences of Schwann cell disruption such as nodal widening and segmental demyelination, axonal degeneration and microvascular lesions (Calcutt, 2004).

The modelisation of diabetes-induced neuropathy is generally made by injection of streptozotocin (STZ) (Courteix et al., 1993; Aubel et al., 2004) or alloxan (Lee et al., 1990) to rats and mice. Those 2 agents that destroy β-cells in the pancreas produce an insulin-dependent diabetes mellitus with marked hyperglycemia.

A single injection of alloxan (40-120 mg/kg i.p.) induces thermal hyperalgesia associated with reduced analgesic efficacy of morphine compared to naïve rats (Ibironke and Saba, 2006; Ahmadi et al., 2012). Injection of STZ at the single dose of 70 mg/kg s.c. or 75 mg/kg i.p. induces not only mechanical and thermal hyperalgesia but also sensitization to chemical stimuli and hyper-responsiveness of C-fibers which persist for at least 4 weeks (Courteix et al., 1993; Aley and Levine, 2001; Chen and Levine, 2003; Aubel et al., 2004). The major problem of this model is that rats also develop physical debility with weight loss, hypolocomotion and major metabolic disorders which complicate data interpretation in studies specifically dedicated to pain. However, much less secondary effects seem to occur when STZ is injected intravenously (50 mg/kg). This route of injection should therefore be preferred especially because hyperalgesia and allodynia are as pronounced but occur earlier than using i.p. or s.c. route (Aley and Levine, 2001).

As a matter of fact, similarities of these rodents models with the human pathology extend beyond chronic peripheral pain since they also include increased vascular permeability and inflammation, vulnerability to nerve ischemia, decrease in nerve conduction velocity, loss of motor and sensory fibers, axonal shrinkage and demyelination (see Aubel et al., 2004). In

addition, extensive pharmacological studies showed that alloxan- or STZ-induced neuropathic-like pain, as assessed by measurement of hyperalgesia and allodynia with validated tests, responds to antidepressants, in particular tricyclics, dual inhibitors of norepinephrine and serotonin reuptake such as paroxetine, duloxetine, venlafaxine and milnacipran (Aubel et al., 2004; Wattiez et al., 2011), and anticonvulsants (such as gabapentin and pregabalin) (Field et al., 1999) but is relatively resistant to opioidergic analgesics, like that observed in diabetic patients suffering from neuropathic pain.

IV.1.b. Anti-retroviral drugs and HIV-related pain

AIDS patients suffer from neurological disorders, notably the neuropathic pain called distal sensory polyneuropathy (So et al., 1988; Schifitto et al., 2002; Estanislao et al., 2004) which affects one third of the patients at advanced stages (So et al., 1988; Simpson and Tagliati, 1995; Childs et al., 1999; Geraci and Simpson, 2000; Schifitto et al., 2002; Finnerup et al., 2005). Distal sensory polyneuropathy is especially refractory to analgesic drugs (Martin et al., 2000; Finnerup et al., 2005). In particular, morphine, which relieves most pains from other origins, can even be pronociceptive in HIV neuropathic patients (Smith, 2011). On the other hand, only few reports mention some improvement of the pain status with treatments aimed at HIV viral suppression (Martin et al., 2000; Herzberg and Sagen, 2001; Bhangoo et al., 2009; Maratou et al., 2009). Indeed, antiretroviral drugs have toxic effects on nerves. This is the case with didanosine, 2',3'-dideoxycytidine and stavudine which cause peripheral neuropathy (Milligan et al., 2000; 2001a; 2001b; Williams et al., 2001; Cherry et al., 2003) and even increase distal sensory neuropathy.

In animal models aimed at studying HIV-induced neuropathy, the glycoprotein gp120, which is a major component of the HIV envelope, is injected either directly onto the sciatic nerve (Herzberg and Sagen, 2001; Jolivalt et al., 2008; Bhangoo et al., 2009; Maratou et al., 2009), intrathecally (Milligan et al., 2000; 2001a; 2001b) or into plantar paw (Jolivalt et al., 2008a). In rodents, these administration modes induce neuropathic pain with mechanical allodynia and thermal hyperalgesia which persist from hours (intrathecally) to weeks (onto the sciatic nerve).

On the other hand, the potency of antiretroviral drugs to induce neuropathic pain has been demonstrated through their administration using intravenous or intraperiteonal route (Joseph et al., 2004; Huang et al., 2013). In those cases, pain, which can last for at least 3 weeks, develops rapidly after a single administration of these drugs. Thus, van Steenwinckel et al. (2008) showed that thermal allodynia as well as mechanical hyperalgesia and allodynia are already striking four

days after a single i.v. injection of 2',3'-dideoxycytidine in rats. Interestingly, similar neuropathic-like pain symptoms develop after systemic administration of this drug in wild-type mice but not in knock-out mutants deficient in the 5-HT_{2A} subtype of serotonin receptors, suggesting that this receptor, whose activation contributes to facilitate NMDA receptor-mediated glutamatergic neurotransmission, plays a permissive role in 2',3'-dideoxycytidine-induced neuropathic pain (van Steenwinckel et al., 2008). Furthermore, evidence has been reported that this anti-retroviral drug produces marked alterations in mitochondrial functions in DRG neurons, but not in spinal neurons, thereby supporting the inference that neuropathic pain results from injury to the peripheral nervous system (van Steenwinckel et al., 2008).

Rather than administering anti-retroviral drugs alone in healthy rodents, a treatment more relevant to the clinics has consisted of the combined administration of gp120 (to mimic HIV-induced neuropathy), directly to the sciatic nerve, and 2',3'-dideoxycytidine, via a systemic (intraperitoneal) route. A clear-cut exacerbated neuropathic pain that markedly exceeds those produced by each treatment alone has been evidenced in rats after such combined treatment (Wallace et al., 2007). Marked alterations in DRG cell phenotypes and microglia activation contribute to neuropathic pain because treatment with the microglial inhibitor, minocycline, has clear-cut efficacy to reduce hypersensitivity to mechanical stimuli by combined gp120-2',3'-dideoxycycline treatment. Interestingly, gabapentin and morphine, but not the tricyclic antidepressant amitriptyline, also reduced gp120 + 2',3'-dideoxycycline-induced neuropathic pain (Wallace et al., 2007), indicating a different sensitivity to drugs as compared to diabetes-induced neuropathic pain (see above).

IV.1.c. Postherpetic neuralgia

Usually, the Herpes zoster virus, which first infection causes varicella, remains dormant in sensory ganglia. However, reactivation and transport of the virus from skin to sensory ganglia can cause Herpes zoster (shingles) (Hope-Simpson, 1965; Dworkin and Portenoy, 1996), particularly in old and/or immunodepressed patients. Acute Herpes zoster induces a rash which is accompanied by pain in the dermatome of the sensory ganglia infected by the virus. Postherpertic neuralgia, which is thought to be induced by nerve damage caused by the virus, can be severe, not responsive to classical analgesic treatments and persistent for years (Dworkin and Portenoy, 1996).

Modelisation of Herpes-evoked pain in rodents is done using the varicella zoster virus itself (Sadzot-Delvaux et al., 1990; Fleetwood-Walker et al., 1999). Usually, rats are injected

subcutaneously with CV-1 cells infected ($4x10^6$ infected cells; 50 µL) with the varicella zoster virus. They develop mechanical allodynia and thermal hyperalgesia within 5 days post infection, and both neuropathic pain-like symptoms last for at least two months, together with the presence of active virus in DRG neurons (Sadzot-Delvaux et al., 1990; Zhang et al., 2011).

Another virus of the same herpes viridae family that has been used for the modelisation of postherpertic pain in rodents is the Herpes simplex virus (HSV-1). Injection of the HSV-1 complex ($1x10^6$ plaque forming units) into the skin hindpaw of mice causes not only allodynia and hyperalgesia but also skin lesions like in humans (Takasaki et al., 2000). The pain behaviors start 5 days post-injection and persist for at least 8 days. HSV-induced neuropathic pain has also been reported in rats injected with HSV-1 (10^7 plaque forming units) into glabrous skin (Dalziel et al., 2004).

The use of those viruses requires special rooms and specific qualifications, which can make these models rather difficult to set up in common laboratories.

Interestingly, acute treatment with resiniferatoxin, an ultra potent TRPV1 agonist, has been reported to reproduce at least some of the characteristics of postherpetic neuralgia in rats, therefore avoiding the special set ups for the use of viruses. A single i.p. injection of resiniferatoxin at the dose of 200 µg/kg induces a reduction in thermal sensitivity but a marked increase in mechanical sensitivity, as shown by profound and persistent tactile allodynia (Baron and Saguer, 1993; Pan et al., 2003). These changes are associated with a marked loss of unmyelinated fibers and extensive ultrastructural damage of myelinated fibers in the sciatic nerve of resiniferatoxin-treated rats (Pan et al., 2003). Furthermore, abnormal sprouting and connections of myelinated primary afferent fibers within the dorsal horn of the spinal cord (notably in lamina II) have also been noted, which might also contribute to resiniferatoxin-induced mechanical allodynia. Because both a reduced thermal sensitivity and a profound tactile allodynia also occur in the dermatomes affected by postherpetic neuralgia in patients (Baron and Saguer, 1993), systemic treatment of resiniferatoxin has been proposed as a model for understanding physiopathological mechanisms underlying this chronic painful condition (Pan et al., 2003).

IV.1.d. Neuropathic pain caused by anti-cancer drugs

Chemotherapy is most frequently used for breast, lung and gastro-intestinal cancers. Because of their neurotoxic effects affecting essentially peripheral nerves, anti-cancer drugs do not reduce cancer-evoked pain but even cause additional neuropathic pain which contributes to major deterioration of the quality of life of treated patients.

Several models using the different anti-cancer treatments have been developed in rodents with the dual aim of understanding underlying physiopathological mechanisms and setting up appropriate anti-pain treatments.

- *Anti-cancer drugs of the taxane family*

Relevant studies have notably been performed with paclitaxel also known as Taxol®. This drug induces impairments of myelinated fiber function at the origin of sensory neuropathy mostly characterized by tingling, numbness, mechanical allodynia, cold allodynia and on going spontaneous burning pain.

Peripheral neuropathy can be induced in rodents by repeated i.p. injections of paclitaxel at the dose of 0.5-1 or 2 mg/kg, four times with two-day intervals. This protocol induces cold allodynia and long lasting mechanical allodynia, reproducing the same features as those seen with early usage of paclitaxel in humans (Polomano et al., 2001; Flatters and Bennett, 2006). When injected daily at the dose of 2 mg/kg during 5 days, rats also develop heat hyperalgesia (Nieto et al., 2008) with a maximum on day 10 after the first injection. Mechanical allodynia usually disappears within 21 days but cold allodynia still persists even after 4 weeks. Neither systemic toxicity nor motor impairments have been reported in rodents after such treatments.

Higher dosages of paclitaxel such as those achieved with either 16 mg/kg i.p. of the drug once a week for 5 weeks, two intravenous injections at 18 mg/kg with a 3 day interval or a single i.p. injection at 32 mg/kg induce thermal hypoalgesia in addition to the other symptoms already noted in low-dosage models (Authier et al., 2000; Kilpatrick et al., 2001). However, systemic toxicity of paclitaxel at high dosage interferes with behavioral tests to assess nociceptive functions, which explains why low dosage is usually preferred in animal studies.

Some studies have also been performed with docetaxel, another anti-cancer drug of the taxane family. Docetaxel injected i.v. once a week for five weeks increases thermal sensitivity and causes degeneration of skin nerves in the foot pad in rats (Roglio et al., 2009).

- *Platinum salts: cisplatin and oxaliplatin*

Cisplatin and oxaliplatin are the most commonly used anti-cancer drugs of the platinum salts family. Repeated administrations of low doses of cisplatin (1 mg/kg i.p. once weekly for 9 weeks or 2 mg/kg i.p. twice a week for 5 weeks) in rats induce behavioral, anatomical and electrophysiological changes similar to those observed in humans treated with this drug. In particular, cisplatin-treated rats exhibit mechanical and cold allodynia as well as thermal hyperalgesia (Cavaletti et al., 1992; Gregg et al., 1992; Authier et al., 2003a; Bianchi et al., 2007; Vera et al., 2007).

Oxaliplatin is less toxic than cisplatin on peripheral organs and functions but is particularly toxic on sensory nerves. Neurotoxicity induced by oxaliplatin is reversible and characterized by dysesthesia and paresthesia. Injections of oxaliplatin at 1-4 mg/kg i.p. twice weekly for 4-5 weeks induce mechanical and cold allodynia and hyperalgesia, as well as thermal heat hyperalgesia with no signs of motor dysfunction (Ling et al., 2007a). To fit better with the clinics in humans, notably for the treatment of the metastatic colorectal cancer, a model that consists of a single administration of oxaliplatin has been developed with different doses: 3, 6 and 12 mg/kg i.p. This treatment induces both mechanical allodynia and hyperalgesia as well as cold allodynia and hyperalgesia, but no heat hyperalgesia or allodynia (Ling et al., 2007b). This model essentially replicates the clinical signs seen in humans after a single injection which are cold allodynia and hyperalgesia.

Another treatment which consists of 3 injections of oxaliplatin at 10 mg/kg i.p. with 3 days intervals between them was recently developed to induce mechanical and thermal allodynia at both extracephalic and cephalic levels in rats (Michot et al., 2013). Data reported using this model further confirmed that neuropathic pain involving trigeminal versus spinal mechanisms has different pathophysiological features and responds differentially to alleviating drugs. In particular, morphine at a low dose (3 mg/kg s.c.) completely reversed oxaliplatin-induced increase in nocifensive responses due to subcutaneous injection of formalin into a hindpaw (extracephalic) territory but was only marginally active against oxaliplatin-induced neuropathic like pain at cephalic (vibrissal pad) level (Michot et al., 2013). No sign of microglial activation could be detected in the spinal cord of oxaliplatin-treated rats, indicating that this neuropathic pain model involves physiopathological mechanisms distinct from those evoked by physical nerve lesion (see below page 36). Indeed, oxaliplatin does not induce direct axonal lesions but causes a decrease of the size of somas and nucleus of DRG neurons and atrophy of intraepidermal fibers (Jamieson et al., 2005; Meyer et al., 2011). Thorough examinations of receptor channels of the TRP family also indicated a modest but significant over-expression of TRPA1 in DRG, which very probably contributes to supersensitivity to mechanical, cold and chemical/inflammatory stimuli in rodents rendered neuropathic by sub-chronic administration of oxaliplatin (Descoeur et al., 2011; Michot et al., 2013).

-*Vincristine*

In humans, vincristine is used notably to reduce breast cancer, and primary brain tumors. It induces neuropathy symptoms especially paresthesia and thermal hyperalgesia,
in all patients (Pal, 1999).

Also in rats, vincristine injected daily at the dose of 75 µg/kg i.v for up to 9 days induces the

development of mechanical hyperalgesia and allodynia with maxima on the 11th day (Aley et al., 1996; Sweitzer et al., 2006). An alternative to this treatment consists of 5 administrations of vincristine at the dose of 150 µg/kg i.v. every other day. This treatment schedule produces mechanical and thermal hyperalgesia and allodynia correlated with electrophysiological alterations and histopathological changes of myelinated nerve fibers without altering motor performance and general physiological status (Authier et al., 2003b). Neuroanatomical studies clearly showed that vincristine-induced neuropathic pain is not associated with any loss of DRG cells or degeneration of proximal sensory axons. However, vincristine-treated rats show marked alterations in mitochondria, characterized by a swelling and disappearance of cristae in lumbar DRG, like those observed with other neuropathic pain-inducing anti-cancer drugs such as paclitaxel (Thibault et al., 2008). A causal relationship could be evidenced between mitochondria alterations and neuropathic pain, notably from data obtained with L-acetyl carnitine, which reverses, at least partially, mitochondrial dysfunction, and can prevent and partly reverse chemotherapy-induced neuropathy (Xiao and Bennett, 2008).

IV.2. Models of peripheral nerve injury

- **At hindlimb level**

IV.2.a. Nerve section

Sciatic nerve section

Unilateral transection of the sciatic nerve at mid thigh level is the first nerve lesion model that has been developed (Wall et al., 1979). In anesthetized rats, the sciatic nerve is exposed and two nylon sutures are tightly tied 1 cm apart, close to its bifurcation (Fig. 6). The nerve is transected twice between the ligatures so as to remove a 5 mm fragment thereby preventing any re-connection. Indeed, a neuroma develops at the proximal nerve stump as a result of regenerative nerve sprouting in all directions. With this procedure, the adjacent saphenous nerve is also lesioned, which leads to the complete denervation of the distal hindlimb. This nerve lesion is considered as an approximate model of “anesthesia dolorosa” in humans (Wall et al., 1979) in which there is also an absence of any sensory input in the innervated area. Among the neuroplastic changes induced within the ipsilateral dorsal horn of the spinal cord in sciatic nerve-sectioned rats, a marked induction of CCK-B receptors of cholecystokinin (CCK) has been reported, especially within the superficial layers (Antunes Bras et al., 1999). Since CCK-B receptor signaling has clear-cut anti-opioid effects, this adaptive change might explain, at least in

part, why morphine is only poorly effective against neuropathic pain in such lesioned rats. Although this sciatic nerve section model undoubtedly contributed to a better knowledge of physiopathological mechanisms underlying neuropathic pain, it has also major limitations since it can lead to autotomy. Whether autotomy reflects pain or just an abnormal sensation or dysesthesia is a matter of debate (Coderre et al., 1986; Blumenkopf and Lipman, 1991; Kauppila, 1998), but, whatever the answer, this dramatic behavioral disorder caused by nerve section raised serious concerns for obvious ethical reasons (Riopelle, 1992). Another major criticism against this model is that complete section of a nerve is relatively uncommon in humans. Accordingly, pathophysiological features in such lesioned animals might not be relevant to those associated with partial nerve lesions which are most frequently observed in patients.

Tibial and sural nerves transection

In this model, tibial and sural nerves are tightly ligated with 5-0 silk thread and sectioned at the distal side of the ligation to remove 2 mm of the distal part of the nerves. In rats, this surgical operation produces mechanical allodynia, cold allodynia and behavioral signs of spontaneous pain of greater amplitude than common peroneal nerve ligation (Lee et al., 2000).

IV.2.b Nerve ligation, compression and other lesion procedures

Complete ligation of the sciatic nerve

The model of chronic constriction injury (CCI), first developed by Bennett and Xie (1988) in the rat, consists of the placement of 4 ligatures with silk (4.0) or chromic gut thread, approximately 1 mm apart, on one sciatic nerve (Fig. 7). The ligatures are loosely tightened until a twitch is observed at the footpad. The purpose of this controlled procedure is to reduce the epineural blood flow without stopping it (Bennett and Xie, 1988; Muthuraman et al., 2008). It results in sciatic nerve swelling, loss of axons distal to the ligatures and neuroma formation at the level of the ligatures. The use of chromic gut tends to increase pain behavior because it promotes the inflammatory immune component of the neuropathy (Maves et al., 1993). Unilateral CCI to the sciatic nerve induces mechanical allodynia and hyperalgesia as well as thermal (heat) hyperalgesia which reach a plateau 2 weeks after the surgery and persists for at least 7 weeks thereafter (Bennett and Xie, 1988; Dowdall et al., 2005; Latrémolière et al., 2008). Cold

hyperalgesia has also been reported in sciatic nerve-ligated rats (Vierck et al., 2005). Even though initial reports indicated hyperalgesia and allodynia only on the lesioned side, more recent reports noted the occurrence of bilateral pain (Spataro et al., 2004; Milligan et al., 2005; Hutchinson et al., 2008). In addition to exacerbate provoked pain behavior, this model induces mild to moderate autotomy and altered posture. Although the pain relevance of such behavioral changes is still a matter of debate (Mogil et al., 2010), the expected postural avoidance of placing body weight on the injury side (guarding) has been documented (Imamura and Bennett, 1995). As a matter of fact, the sciatic nerve ligation model undoubtedly replicates most symptoms of the complex regional pain syndrome in patients (Bennett and Xie, 1988). However, because of difficulties inherent to the control of constriction tightness (Ro and Jacobs, 1993) and variations linked to the choice of suture material (Maves et al., 1993), some variability in sciatic nerve ligation-evoked neuropathic pain-like symptoms is unavoidable from one operated rat to another.

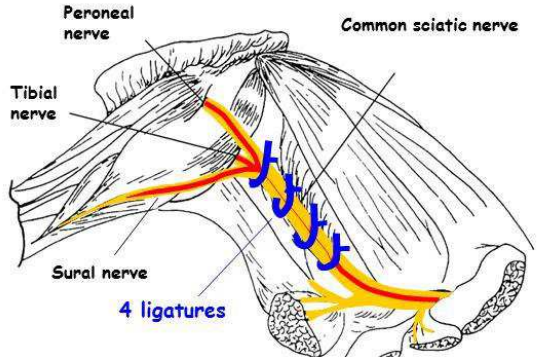

Figure 7:
Model of chronic constriction injury to the sciatic nerve
(Bennett and Xie, 1988)

Partial ligation of the sciatic nerve

In 1990, Seltzer et al. developed a model that consists of unilateral tight ligation of one-third to one half of the sciatic nerve with a 8-0 silk suture just distal to the point at which posterior biceps semitendinous nerve branches off. Spontaneous pain, as suggested by guarding behavior of the ipsilateral hindpaw and frequent licking, together with cold allodynia, chemical hyper-reactivity and mechanical hyperalgesia are induced from 1 to 6 weeks after surgery (Mitchell et al., 1999; Dowdall et al., 2005; Yi et al., 2011). Because of the rapid onset and long lasting continuation of touch-evoked allodynia and hyperalgesia, the partial sciatic nerve injury model is generally considered as mimicking causalgiform pain disorders in humans. In support of this assertion, mechanical hyperalgesia occurs bilaterally in rats with unilateral partial sciatic nerve ligation, like the “mirror image” pains reported by patients suffering from causalgia with excessive sympathetic activity.

Sciatic nerve cuffing

Placement of a polyethylene cuff around the sciatic nerve has been developed as an alternative to nerve ligation, with the idea of reducing the inter-individual variability noted above. Indeed, neuropathic pain similar to the one produced by the CCI model of Bennett and Xie (1988) is induced by a polyethylene cuff (2 mm in length, inner diameter 0.7 mm) placed around the common branch of the sciatic nerve in rats. Heat hyperalgesia and mechanical allodynia, which last for 3 weeks and 2 months respectively, have been reported in rats and mice with sciatic nerve cuffing (Mosconi and Kruger, 1996; Pitcher et al., 1999; Benbouzid et al., 2008a) . Comparison with the sciatic nerve ligation model seems to confirm that the cuffing model leads to less variability in the intensity of neuropathic pain-like symptoms from one operated rat to another (Benbouzib et al., 2008c; Barrot, 2012).

Spared nerve injury

Another variant of the Bennett and Xie's model consists of tight ligation of 2 of the three branches of the sciatic nerve, the tibial and the common peroneal nerves, with 5-0 silk thread followed by axotomy of a 2 mm nerve segment between ligatures. The third branch, e.g. the sural nerve, is spared (Decosterd and Woolf, 2000). This model induces mechanical allodynia as well as hot and cold allodynia at ipsilateral hindpaw from 1 week to 6 months post-injury (Decosterd and Woolf, 2000; Bourquin et al., 2006).

Laser induced sciatic nerve injury

In this model, rodents are first treated acutely with erythrosine B (32.5 mg/kg i.v.), a photosensitizing dye laser. The sciatic nerve is then exposed and irradiated with an argon ion laser to trigger a photochemical reaction that causes thrombosis and occlusion of small vessels supplying the nerve. This results in long lasting bilateral mechanical allodynia and unilateral thermal hyperalgesia (Gazelius et al., 1996; Hao et al., 1999). Ipsilateral pain is induced more rapidly and has a greater magnitude than that generated on the contralateral side. After such laser-induced ischemic nerve lesion, approximately 95% of the animals not only develop neuropathic pain but show also clear-cut behavioral signs of spontaneous pain (Kupers et al., 1998).

Laser beam, on its own, can also induce neuropathic pain. Thus, without any photosensitizing dye, irradiation of the sciatic nerve by a 532 nm laser beam of 1 mm in diameter at an ouput power of 100 mW for 30 seconds results in a marked reduction of the blood flow to the nerve, ending with neurodegeneration (Chiang et al., 2005). Under these conditions, rats develop thermal hyperalgesia and mechanical allodynia which last for 4 to 9 weeks after the surgery.

Sciatic nerve cryoneurolysis

Cold-induced lesion of the sciatic nerve has also been proposed as a model for generating neuropathic pain in rats. According to Willenbring et al. (1995), the sciatic nerve is frozen proximal to its trifurcation with a cryoprobe cooled at -60°C using a 2-mm diameter cryoprobe with nitrous oxide as the refrigerant (30 sec/5 sec/30 sec freeze/thaw/freeze cycle). This operation induces bilateral mechanical allodynia for up to 21 days (Deleo et al., 1994; Willenbring et al., 1995; Colburn et al., 1999), but no thermal hyperalgesia. Interestingly, sympathectomy does not reduce cryoneurolysis-induced mechanical allodynia, in contrast to that found in case of mechanical allodynia in rats rendered neuropathic by spinal nerve ligation or partial ligation of the sciatic nerve (Willenbring et al., 1995), further indicating that freeze-induced lesion probably triggers structural and functional alterations of nociceptive signaling pathways different from those resulting from mechanical lesion of nerves. In line with such differences, autotomy seems to occur more frequently after sciatic nerve cryoneurolysis, which explains why this model did not give rise to extensive studies.

Common peroneal nerve injury

This lesion model (Fig. 7) has been elaborated in the mouse. It consists of a single ligature of the common peroneal nerve with chromic gut suture 5-0 (Vadakkan et al., 2005). The common peroneal nerve has been chosen because one of its branches, the superficial peroneal nerve, carries mainly sensory fibers from the dorsal part of the foot. The nerve is slowly tightened until contraction of the foot dorsiflexors. This model is characterized by allodynia and thermal hyperalgesia without apparent alterations in motor functions. It therefore contrasts with most other neuropathic pain models in which both sensory and motor nerve fibers are lesioned, thereby directly causing abnormal sensory and motor responses.

- - At other levels

Dorsal rhizotomy

This model has been extensively characterized by Lombard et al. (1979). It consists of unilateral sections of lumbar dorsal roots. Briefly, a dorsal hemilaminectomy is performed in deeply anesthetized rats and, through an opening of the dura matter, nine dorsal roots (T13-S2) are sectioned intradurally, proximal to the spinal cord, with great caution to avoid trauma to spinal tissues and ventral roots. This surgery results in mechanical allodynia (Colburn et al., 1999) and mechanical and cold hyperalgesia (Lombard et al., 1979; Ramer et al., 2004), which resemble the symptoms of nerve root avulsions in humans (Bruxelle et al., 1988).

Spinal nerve ligation

In this model developed by Kim and Chung (1992) also in the rat, L5 and L6 spinal nerves are unilaterally ligated under deep anesthesia using 6-0 silk suture (Fig. 8). This results in mechanical allodynia, cold allodynia and thermal heat hyperalgesia. Spontaneous pain can also be observed in operated animals with ipsilateral hindlimb in guarding position (Kim and Chung, 1992). These behavioral manifestations start to develop within 48 h after spinal nerve ligation and last for 10-16 weeks (Kim and Chung, 1992; Choi et al., 1994; LaBuda and Little, 2005). Because of these well established characteristics, the spinal nerve ligation model is frequently used in studies aimed at investigating physiopathological features and potential therapeutic strategies relevant to causalgia resulting from peripheral nerves injury in patients.

Injury to dorsal root ganglia

Rather than nerves, dorsal root ganglia can also be injured to generate experimental neuropathic pain. Thus, Liu et al. (2002) inserted a small stainless steel rod (4 mm in length and 0.5-0.8 mm in diameter) into the L5 and/or L4 intervertebral foramen to produce foramen stenosis. The resulting chronic steady compression of L5/L4 dorsal root ganglia induced heat hyperalgesia, as well as mechanical hyperalgesia and allodynia underlain by increased excitability of ganglion neurons with lowered threshold currents and action potential voltage thresholds as well as increased incidence of repetitive discharges (Song et al., 1999, 2003; Zhang et al., 1999b). Both hyperalgesia and allodynia on the one hand and electrophysiological changes on the other hand could be prevented/reversed by NMDA receptor antagonists, indicating their mediation through spinal glutamate neurotransmission (Song et al., 2003).

Infraorbital nerve ligation

The model of chronic constriction injury to the infraorbital nerve, first developed by Vos et al., (1994) in the rat, consists of the placement of 2 ligatures with silk (4.0) or chromic gut thread, approximately 1 mm apart, on one infraorbital nerve (Fig. 9). Rats developed abnormal spontaneous pain-related behavior (Kryzhanovski et al., 1991, 1993), thermal heat hyperalgesia (Kryzhanovski et al., 1991; Imamura et al., 1997) and mechanical allodynia located at the vibrissae pad 2 weeks after the surgery (Vos et al., 1994; Idänpään-heikkilä and Guilbaud, 1999, Latremolière et al., 2008). The purpose of this model is to reproduce and decipher the pathophysiological mechanisms of typical cephalic pain such as “cluster headache” in humans.

Interestingly, Kayser et al. (2002) showed that, in rats, pain resulting from infraorbital nerve ligation can be reduced by antimigraine drugs such as triptans in contrast to neuropathic pain generated by sciatic nerve ligation.

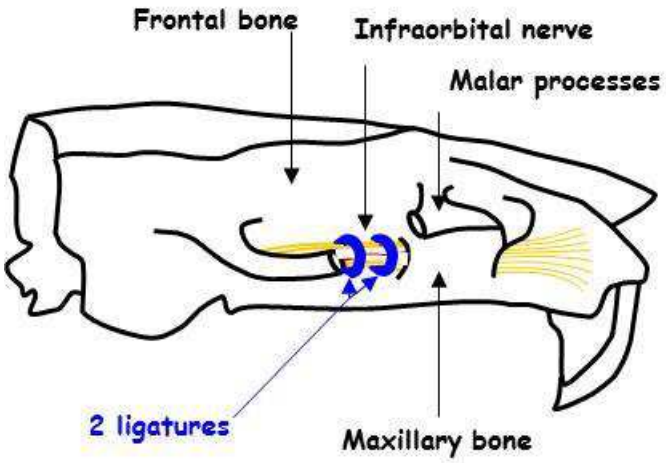

Figure 9:

Chronic constriction injury to the infraorbital nerve.

(Vos et al., 1994)

IV.3. Models of spinal cord injury (SCI)

Spinal cord contusion

It is the oldest and still the most widely used model of SCI. It consists of the contusion of the spinal cord with an impactor such as a weight drop impactor. Under deep anesthesia, spinal cord is exposed after laminectomy and injured by dropping a rod from a specified height. The resulting lesion induces severe paraplegia above-, at- and below-impact level (Hulsebosch et al., 2000; Crown et al., 2006; Nakae et al., 2008). Because cervical contusion can be life threatening through dramatic consequences on heart or lung functions, contusion is usually made at thoracic or lumbar level only. Thermal hyperalgesia (Hoschouer et al., 2009) and mechanical hyperalgesia and allodynia (Kerr and David, 2007) have been described in spinal cord lesioned rats. However, a large interindividual variability exists in this regard because not all of the rats develop pain behavior (Crown et al., 2012). Indeed, unavoidable differences in the extent and location of the spinal cord damage induced by the rod in one rat to another very probably account for this high degree of variability (Basso et al., 1996).

Clip compression injury

Under deep anesthesia, rats undergo a laminectomy and a moderate to severe incomplete injury to the spinal cord is made by a clip compression of 35 g to 50 g of the cord during 1 min, usually

at the thoracic level (T13). After such procedure, rats develop mechanical allodynia and hyperalgesia at-, below- and above- the site of injury (Bruce et al., 2002; Densmore et al., 2010), as well as thermal and cold hyperalgesia below the level of injury (Hama and Sagen, 2007) which can be reduced by amitriptyline, morphine and gabapentin, all drugs used for alleviating neuropathic pain in patients with spinal cord injury. These pharmacological data support the idea that this spinal cord injury model in rats fills most of the "face", "construct" and "predictive" validities expected for reliable assessment of the potential therapeutic value of innovative drugs aimed at reducing SCI-evoked pain in patients.

Spinal cord transection

Under deep anesthesia, rats undergo a laminectomy, and the spinal cord is transected usually at the thoracic level (T8-T9). A gel foam is inserted between the two ends in order to reduce bleeding and to control that transection is complete. This model has notably been used to study specifically the central pattern generator at lumbar level (below the lesion) which controls extensor and flexor muscles involved in locomotor movements of hindlimbs (Antri et al., 2003, 2005). In addition, it has allowed thorough investigations of pathophysiological mechanisms underlying spasticity and hyper-reflexia which are classical symptoms in paraplegic and tetraplegic patients (Ko et al., 1999; Boulenguez et al., 2010; Murray et al., 2010; Yates et al., 2011). Regarding pain, nociceptive messages generated below the lesion no longer reach the brain, and this model can be used only for studying above- and at-level neuropathic pain consecutive to the transection. Indeed, rats with complete transection of the spinal cord do not develop above-level neuropathic pain but a strong at-level neuropathic pain is triggered for months with a maximum 4 weeks after the surgery (Scheifer et al., 2002; Hoheisel et al., 2003; Densmore et al., 2010). Long lasting mechanical allodynia is particularly pronounced in cutaneous territory around the transection, together with increased neuronal activity in the 2-3 spinal segments located just rostrally (Hoheisel et al., 2003). Although complete lesion of the spinal cord is rather rare after injury in humans, the model of complete transection in rats has some translational relevance because the resulting at-level mechanical allodynia is responsive to drugs used for alleviating pain in paraplegic and tetraplegic patients.

A variant of this model consists of spinal cord hemisection, which also produces mechanical hyperalgesia along with mechanical and cold allodynia (Hains et al., 2003; Kim et al., 2009). However it suffers from interindividual variability because of unavoidable variations in the location and extent of lesion from one rat to another.

Spinal cord ischaemia

The technique used for lesioning spinal cord tissues by laser-mediated ischemia is very close to the one described above for the sciatic nerve. The first step also consists of the i.v injection of a photosensitizing dye such as erythrosin B. Then, an argon ion laser beam is directly focused at vertebrae level. The resulting blood vessel occlusion generates ischemia-induced spinal cord injury (Watson et al., 1986). This model has the great advantage to avoid laminectomy, which allows studies of the effects of spinal cord injury *sensu stricto*, and its intrinsic role in the resulting pain. Mechanical as well as cold allodynia at level- and below- the lesion have been regularly reported after such laser-induced spinal cord lesions in rats. Furthermore, evoked mechanical and thermal allodynia are both responsive to drugs which are effective to alleviate neuropathic pain in spinally lesioned patients (Watson et al., 1986; Hao et al., 1991, 1992, 2006), as expected of a relevant model for assessing the potential value of innovative treatments targeting this type of central pain.

Neuropathic-like pain evoked by chemicals administered at the spinal level

Intrathecal administration of ATP

ATP is abundantly released after neural injury, not only by the damaged cells but also by astrocytes and microglia within the spinal cord. The role and the importance of purinergic signaling in neuropathic pain has been extensively reviewed in the literature (Burnstock, 2009; Inoue and Tsuda, 2012), emphasizing that activation of ATP receptors, especially the ionotropic P2X4 and P2X7 types on activated microglia, is critical for the induction and maintenance of pain through the release of numerous synapse-sensitizing factors and activation of downstream signaling pathways (Tsuda et al., 2003; Honore et al., 2006). Accordingly, the direct intrathecal injection of ATP itself (30, 100, 300 nmol/10 µL) or of the P2X agonist α-β-Me-ATP (10, 30 nmol/10 µL) induces mechanical allodynia which persists for 1-3 weeks after acute administration (Nakagawa et al., 2007). Thermal hyperalgesia has also been reported after intrathecal injection of α-β-Me-ATP (30 µg/5 µL) as well as 2-methylthio-ATP (3 and 30 µg/5 µL) (Tsuda et al., 1999). Physiopathological mechanisms downstream of P2X receptor activation involve notably the release of pro-inflammatory chemokines (CXCL2) and cytokines (IL-1β) like those triggered by neural lesion causing neuropathic pain, in support of the idea that acute intrathecal administration of ATP has some interest to model neuropathic pain in animals.

Intrathecal administration of BDNF

Whereas BDNF seems to have an analgesic role in midbrain (Siuciak et al., 1994, 1995), its contribution to neuropathic pain states at the spinal level has been clearly demonstrated in several convergent studies (Thompson et al., 1999; Marcol et al., 2007; Wang et al., 2009; Trang

et al., 2011). Accordingly, intrathecal injection of BDNF (50 ng/25 µL) induces both in rats and mice long lasting thermal hyperalgesia and mechanical allodynia (Yajima et al., 2005; Zhang et al., 2012). Recently, Constandil et al. (2011) showed that a single injection of a very low dose of BDNF, 0.003 ng/ 10 µL, is sufficient to trigger long lasting mechanical hyperalgesia in the rat. These data further underline that BDNF is a key factor in the induction and maintenance of neuropathic pain after neural lesions (Wang et al., 2009) and support the idea that direct intrathecal administration of this neurotrophic factor produced by various cell types, especially activated microglia and astrocytes, could be an especially relevant paradigm to study underlying physiopathological mechanisms.

Excitotoxic injury to the spinal cord

Spinal cord injury can be made by intraspinal injection of excitotoxic agonists of glutamate receptors such as the AMPA-R agonist quisqualic acid, NMDA-R agonists, ibotenic acid or kainic acid. Indeed, SCI produces dramatic increase in extracellular glutamate concentration, up to excitotoxic level, and the direct intraspinal administration of these agonists in fact triggers physiopathological mechanisms similar to those resulting from other types of lesion, especially mechanical lesion of the spinal cord (Wilcox, 1988; Aanonsen and Wilcox, 1989; Liu et al., 1991). Usually, the reproducibility of lesions induced by direct intraspinal injection of excitotoxic agonists of ionotropic glutamate receptors is higher than after mechanical injury which explains why 100% of treated animals (rats and mice) develop long lasting spontaneous pain, mechanical allodynia and thermal hyperalgesia (Yezierski et al., 1998; Fairbanks et al., 2000).

	Peripheral nerve injury	Mechanical allodynia	Mechanical hyperalgesia	Heat hyperalgesia	Cold hyperalgesia
Sciatic nerve	Sciatic nerve section	●	●	●	●
	Chronic constriction injury	●	●	●	●
	Partial ligation of the sciatic nerve	●	●	●	●
	Sciatic nerve cuffing	●	○	●	●
	Spared nerve injury	●	●	⊗	●
	Sciatic nerve cryoneurolysis	●	○	⊗	●
	Laser induced nerve injury	●	○	●	●
Other neural tissues	Tibial and sural nerve transection	●	○	○	●
	Common peroneal nerve injury	●	○	●	●
	Spinal nerve ligation	●	●	●	●
	Dorsal rhizotomy	●	○	○	●
	Injury to dorsal root ganglia	●	●	●	⊗
	CCI-ION	●	○	●	●

	Drugs and virus induced neuropathic pain	Mechanical allodynia	Mechanical hyperalgesia	Heat hyperalgesia	Cold hyperalgesia
Diabetes induced neuropathic pain	Streptozotocin	●	●	●	●
	Alloxan	●	●	●	●
Anti-cancer induced neuropathic pain	Paclitaxel (taxol)	●	●	●	●
	docetaxel	●	●	●	●
	Cisplatin	●	●	●	●
	Oxaliplatin	●	●	●	●
	Vincristine	●	●	●	●
Anti-retroviral and HIV induced neuropathic pain	Anti retroviral drugs (ddi, ddc)	●	●	●	○
	Gp 120	●	●	●	○
Postherpetic neuralgia	Varicella zoster virus	●	○	●	○
	Hsv1	●	●	●	○
	Resiniferatoxin	●	○	⊗	○

Spinal cord injuries	Mechanical allodynia	Mechanical hyperalgesia	Heat hyperalgesia	Cold hyperalgesia
Spinal cord contusion	●	●	●	○
Clip compression injury	●	●	●	●
Spinal cord transection	●	○	○	○
Spinal cord hemisection	●	○	●	●
Spinal cord ischaemia	●	○	●	●

Chemicals at the spinal level	Mechanical allodynia	Mechanical hyperalgesia	Heat hyperalgesia	Cold hyperalgesia
ATP	●	○	●	○
BDNF	●	●	●	○
Excitotoxic injury	●	○	●	○

Table 3 : Summary of the different neuropathic pain models and their associated symptoms of allodynia and hyperalgesia.

● : presence

○ : not documented

⊗ : absence

CHAPTER V: PHYSIOPATHOLOGICAL MECHANISMS UNDERLYING CENTRAL AND/OR PERIPHERAL NEUROPATHIC PAIN – PHARMACOLOGICAL, CELLULAR AND MOLECULAR DATA

V.1. Pharmacological data.

Most of pharmacotherapeutic treatments of neuropathic pain currently used are based on empirical data. The difficulty of setting up controlled trials, especially for central neuropathic pain, and the lack of mechanism-based treatments leave clinicians in an uncomfortable position, where their only choice is to prescribe one of the most used drugs hoping that it will provide relief for the patient. If this drug does not work, it will be changed to another one until finding the most appropriate for the patient i.e. the one that will provide the better relief with the least harmful effects. It was emphasized more than 10 years ago that rather than treating only by "intuition", patients should benefit therapeutics issued from mechanism-based evidence (Woolf et al., 1998; Woolf and Mannion, 1999). This approach is meant to design treatment according to the patient'symptoms and the origin of his/her pain (Jensen and Baron, 2003), which led to several guidelines (Finnerup et al., 2005; Manchikanti et al., 2009; Attal et al., 2010; Siddall, 2012) aimed at identifying the best medications for each neuropathy type. These guidelines agree on the fact that whatever neuropathic pain, the best treatments are, in first line, antidepressants, such as the tricyclic amitriptyline and the mixed norepinephrine/serotonin reuptake inhibitors duloxetine and venlafaxine, and anticonvulsants like gabapentin and pregabalin (Table 4). The second or third line treatments are opiates, including morphine, tramadol, tapentadol and other compounds acting preferentially at μ opioid receptors (Attal et al., 2010). In addition, voltage-dependent sodium channel blockers, such as lidocaine and bupivacaine, can also be prescribed as adjuvants, especially through local patch applications at painful territories.

Though the same medications are prescribed, the few randomized controlled trials that had been carried on showed that pain resulting from spinal cord lesion is more refractory to treatment than pain evoked by peripheral nerve lesion. Actually, antidepressants are often unsuccessful and/or less tolerated in patients with neuropathic pain from central versus peripheral origin. Indeed, for antidepressants as well as for most anticonvulsants, the number needed to treat (NNT) is higher in central versus peripheral pain conditions (Fig. 10). The difference is even more striking with lidocaine patches which are somewhat efficient to reduce peripheral pain but are inactive against central pain (Siddall and Middleton, 2006; Finnerup et al., 2010).

Interestingly, in rats, blockade of voltage-dependent sodium channels by a microgram dose of tetrodotoxin was found to effectively reverse mechanical allodynia and hyperalgesia evoked by sciatic nerve ligation whereas this treatment was totally ineffective against mechanical allodynia induced by ligation of the infraorbital nerve (Kayser et al., 2010). Therefore, differences in the response to alleviating drugs not only exist between neuropathic pain of peripheral versus central origin, but also between neuropathic pain in cephalic versus extra-cephalic territories (Kayser et al., 2002, 2010, 2011).

V.2 Cellular and molecular data

V.2.a. Loss of inhibitory transmission

GABA plays a pivotal role in the regulation of peripheral and central neuropathic pain through descending and local interneuron GABAergic inhibitory pathways. Loss of GABAergic inhibition and neuropathic pain relief by GABA receptor agonists have been reported after spinal cord injury (Drew et al., 2004; Gwak et al., 2006, 2008) as well as after peripheral nerve injury (Eaton et al., 1999; Moore et al., 2002; Scholz et al., 2005). Lesion-induced decrease in GABAergic inhibition is not only due to lower levels of extracellular GABA but also to a down-regulation of the GABA synthesizing enzymes GAD-65 and GAD-67 (Eaton et al., 1998; Meisner et al., 2010). Transplantation of human neural precursor cells differentiated into GABAergic cells has been reported to reduce neuropathic pain of peripheral or central origin, further supporting the idea that a deficit in GABAergic tone contributes to both types of neuropathic pain in lesioned animals (Eaton et al., 2007; Mukhida et al., 2007; Bráz et al., 2012). Another mechanism which disrupts inhibition in the spinal cord is the change in the expression of the two cation-chloride co-transporters NKCC1 and KCC2. NKCC1 and KCC2 pump chloride ions in and out neurons, respectively. Lesion-induced dysregulation in their expression leads to a shift in Cl^- concentration gradient at cell membrane, and results in reduced hyperpolarization or even a depolarisation by GABA (Coull et al., 2003). KCC2 is notably downregulated in models of peripheral (Jolivalt et al., 2008; Janssen et al., 2012) and central neuropathic pain (Lu et al., 2008b; Boulenguez et al., 2010), thus inducing an elevated intracellular Cl^- concentration. NKCC1 has been shown to have a role in the modulation of inflammatory (Granados-Soto et al., 2005) and central neuropathic pain (Cramer et al., 2008) but its implication in peripheral neuropathic pain remains to be investigated.

V.2.b. Neuroinflammatory processes

Nerve injury induces neurons insult and loss which result in the induction of the transcription factor ATF3 and the release of numerous factors such as ATP and lipopolysaccharides (LPS) or heat shock proteins by injured neurons. Those molecules then activate purinergic receptors and toll-like receptors, respectively (Fig. 11).

Purinergic receptors

P2X4 receptors are induced in microglia and neurons after spinal cord injury or peripheral nerve injury. P2X4 upregulation starts early and lasts for weeks after neural lesion. P2X4 receptor activation by ATP leads to the production of pro-inflammatory cytokines and the release of BDNF (Schwab et al., 2005; Ulmann et al., 2008; Trang et al., 2009; Vaccari et al., 2012). Recently, their implication in neuropathic pain has been further highlighted by the fact that antidepressants block their action, thereby providing an explanation of the anti-neuropathic effects of these drugs (Nagata et al., 2009). P2X7 receptors are also expressed by microglia and are implicated in peripheral neuropathic pain (Honore et al., 2006; McGaraughty et al., 2007). Though the role of the purinergic P2X7 receptor in inflammation after spinal cord injury has been clearly identified, its role in central neuropathic pain remains to be defined (Marcillo et al., 2012).

Toll-like receptors (TLR)

TLR are localised on immune cells and particularly on microglia and macrophages (Laflamme and Rivest, 2001; Zekki et al., 2002). Their activation triggers NFκB signaling pathway, and thus promotes the production and release of pro-inflammatory cytokines (Zhang et al., 1999a; Medzhitov, 2001). After CNS or PNS lesion, cleavage of extracellular matrix products leads to the release of LPS and heat shock proteins which act as agonists of TLR2 and TLR4. TLR activation might be deleterious for the CNS, because the TLR agonists zymozan and LPS activate macrophages. Indeed, after L5 root transection, TLR4 -/- mice display a lower activation of macrophages and a decreased production of cytokines (Tanga et al., 2005). However after SCI, TLRs are essential to regulate gliosis and inflammation. Following spinal cord injury, knock-out mice deficient in TLR4 or TLR2 showed a marked impairment of locomotor activation, an increase in gliosis and myelin sparing compared to wild-type mice implying a potential beneficial role of TLR after spinal cord injury.

Glial activation

As already mentioned above, purinergic receptors and TLR are expressed by glia and microglia, and their stimulation leads to activation of both cells phenotypes (Inoue, 2002, 2006; Lehnardt et al., 2002; Fellner et al., 2013). Microglia plays a key role in the induction and maintenance of neuropathic pain after central or peripheral nerve injury, and its inhibition efficiently reduces neuropathic pain (Latrémolière et al., 2008; Carlton et al., 2009; Tsuda et al., 2013). In contrast, astrocytes may not have the same role in central and peripheral pain. Whereas the implication of activated astrocytes in the initiation and maintenance of peripheral neuropathic pain is clearly established (Garrison et al., 1991; Chiang et al., 2012; Ji et al., 2013). Their role in central

neuropathic pain remains controversial. On the one hand, propentofylline, an inhibitor of astrocyte activation, has been shown to reduce spinal cord injury-induced pain thereby suggesting that astroglial cells contribute to central neuropathic pain (Gwak et al., 2008, 2012). On the other hand, astroglia may be neuroprotective as well. Indeed, after spinal cord injury, an astroglial scar is formed (Eng et al., 1987), which stops axonal growth, therefore preventing any locomotor recovery. Nevertheless, disruption of the astroglial scar results in the widespread invasion of remote spinal areas by inflammatory cells leading to an increase in the lesion volume, which further worsens locomotor function (Okada et al., 2006; Herrmann et al., 2008). This inflammation might also be deleterious for pain, thereby suggesting that astrocytes might have a protective role in SCI.

Cytokines

Glial and microglial activation leads to the release of pro-inflammatory cytokines namely IL-6, IL-1β and TNF-α.

These cytokines are known to induce pain by themselves through p38-mediated central sensitization and disruption of GABAergic inhibitory mechanisms (Liu et al., 2007; Kawasaki et al., 2008; Gwak and Hulsebosch, 2011; Liu et al., 2013). Although pro-inflammatory cytokines are all induced in central and peripheral neuropathic pain, it seems that the spatio-temporal pattern of microglia activation and cytokines-releasing microglia differs between central and peripheral neuropathic pain, highlighting a difference in terms of respective physiopathological mechanisms.

Although spinal cord injury also clearly induces a persistent glial and microglial activation, peripheral nerve lesion seems to trigger a larger production and release of pro-inflammatory cytokines (Detloff et al., 2008).

V.2.c. MAP kinases (Fig. 11)

MAPK family consists of three major members: extracellular signal regulated kinase (ERK, including ERK ½), p38 and c-JUN N-terminal kinase (JNK). They control three different signaling cascades that transduce a broad range of extracellular stimuli into intracellular signals and events (Ji et al., 2009).

ERK

Erk is activated by persistent neural activity and upregulated after nerve injury (Hao et al., 2005). ERK activation is induced by glutamate neurotransmission via NMDA and AMPA receptors (Ji et al., 1999; Kawasaki et al., 2004). One of the main pathways leading to ERK activation is TrkB

receptor activation. Indeed TrkB activation by BDNF contributes to a 40% increase in the density of dorsal horn neurons expressing pERK in response to peripheral noxious stimuli (Pezet et al., 2002b).

pERK has a predominant role in the induction of Long Term Potentiation (LTP). It is required for the phosphorylation and the trafficking of NMDA and AMPA receptors to the plasma membrane (Galan et al., 2004; Slack et al., 2004). Its role is central neuropathic pain has been shown with the prevention of grooming behavior by pharmacological blockade of ERK pathway after excitotoxic spinal cord injury (Yu and Yezierski, 2005). On the other hand, ERK blockade reduces CREB activation and associated thermal and mechanical allodynia in several models of peripheral neuropathic pain (Ma and Quirion, 2005; Song et al., 2005). Although ERK was originally thought to be activated only in neurons, further studies clearly demonstrated that it is also activated in astrocytes and microglia after nerve lesion (Zhuang et al., 2005).

p38

There are four different p38 isoforms: p38α, p38β, p38γ, and p38δ. The isoforms p38α and p38β are the most abundant in the mature nervous system (Ji et al., 2009).In contrast to ERK, p38 is mostly activated by the neuroinflammatory process, notably by cytokines and chemokines (Abbadie et al., 2003; Sung et al., 2005; Svensson et al., 2005). Its activation is correlated with neuropathic pain in models of peripheral and central nervous system injury, and administration of intrathecal p38 inhibitor could reduce both central and peripheral neuropathic pain (Jin et al., 2003; Tsuda et al., 2004; Daulhac et al., 2006; Hains and Waxman, 2006). Once activated, the role of p38 mostly consists of the enhancement of inflammation with increased production of cytokines, iNOS, COX2 as well as PGE2 (Svensson et al., 2003a; 2003b; Sung et al., 2005).

JNK

Whereas ERK is mostly present in neurons and p38 in microglia, JNK is primarily and persistently expressed in spinal cord astrocytes (Zhuang et al., 2006), notably through the stimulatory influence of TNF-α and FGF-2 (fibroblast growth factor 2). JNK activation leads to the release and up regulation of several chemokines such as MCP-1 (Gao et al., 2009, 2010). Its role in *peripheral* neuropathic pain has been inferred from studies showing that intrathecal injection of the JNK inhibitor sp 600125 reduces neuropathic pain (Obata et al., 2004; Daulhac et al., 2006; Zhuang et al., 2006). Eventhough JNK pathway is well known to promote apoptosis and reduce locomotor recovery after spinal cord injury (Nakahara et al., 1999; Yin et al., 2005; Repici et al., 2012), its role in *central* neuropathic pain still remains to be elucidated.

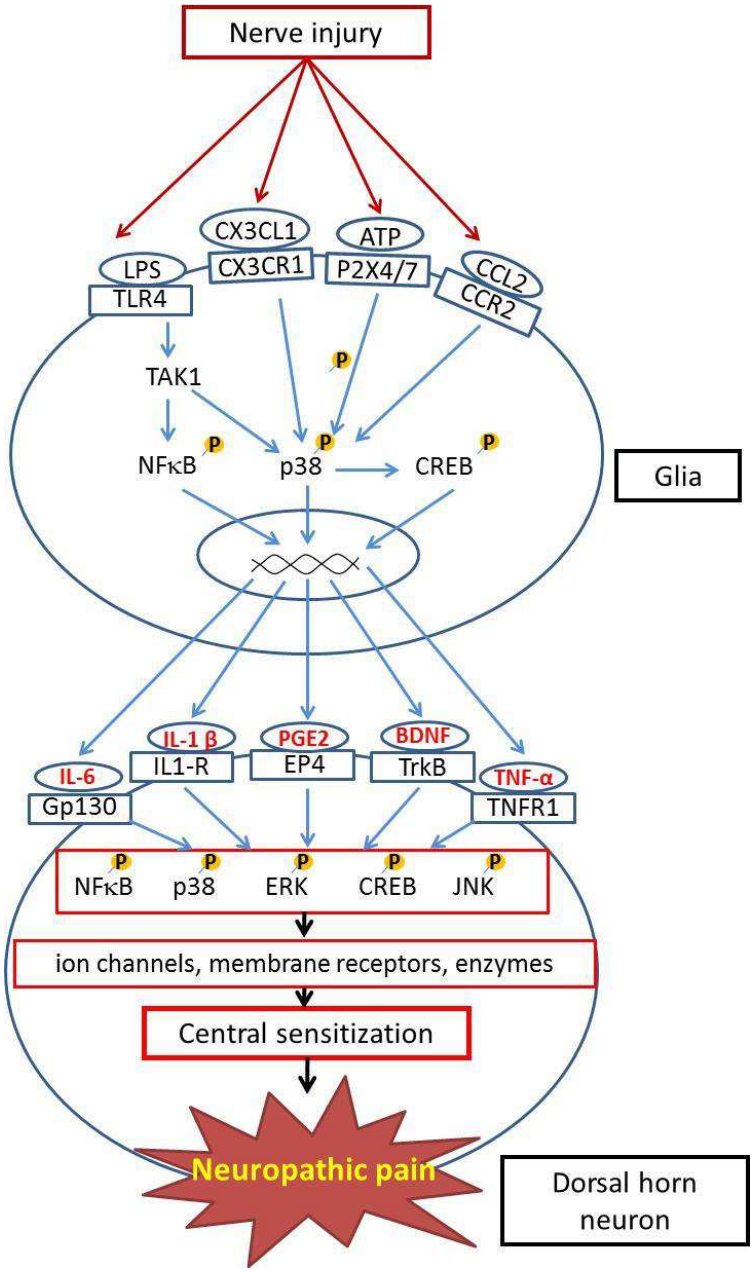

Figure 11: Main signaling pathways involved in neuropathic pain.

Nerve injury triggers the release of factors such as ATP and LPS which induce p38 activation in microglia. In turn, p38 activation promotes the release of cytokines and neurotrophic factors, whose binding to respective receptors (on second order neurons) triggers central sensitization in the spinal cord through kinases pathways (from Schäfers et al., 2003; Miletic et al., 2004 ; Sung et al., 2005 ; Zhuang et al., 2007; Lee et al., 2009; Trang et al., 2009; Chu et al 2010; Clarck et al., 2010 ; Gao and Ji, 2010 ; Liu et al., 2010 ; Cruz Duarte et al., 2012).

PGE2: prostaglandin E2 ; EP4:Prostaglandin E receptor 4; NFκB: nuclear factor kappa B; GP130: glycoprotein 130; TNFR1: TNF receptor 1.

V.2.d. Synaptic plasticity

V.2.d.1 NMDA receptors

NMDA receptor is a receptor-channel activated by glutamate and permeable to Ca^{2+}. Its tetrameric structure generally comprises 2 NR1 subunits and 2 NR2 subunits (NR2A, NR2B, NR2C, NR2D, NR3A, NR3B) (Nagy et al., 2004; Stephenson, 2006). In basal, resting conditions, a Mg^{2+} ion is localized at the receptor, blocking Ca^{2+} influx (Mayer et al., 1984).
In the spinal dorsal horn, NMDA receptors are localized on primary afferents endings and on dorsal horn projection neurons. Prolonged and intense stimulations in the dorsal horn induce the release of glutamate, substance P and CGRP which, in turn, cause a depolarization sufficient to remove Mg^{2+} from its blocking site, thereby allowing Ca^{2+} influx through activated NMDA receptor channel (Mayer et al., 1984). Inside neurons, Ca^{2+}induces the activation of several signaling pathways such as PKC (protein kinase C), CamKII (Ca2+/calmodulin dependent protein kinase II) and ERK pathways, leading to phenotypic modifications of activated neurons (Latremoliere and Woolf, 2009).
These various kinases phosphorylate the NR1 and NR2 subunits of the NMDA receptor and contribute to neuropathic pain states notably in the spinal cord (Guo et al., 2002, 2004; Zou et al., 2002; Petrenko et al., 2003; Brenner et al., 2004; Katano et al., 2011; Zhou et al., 2011). Particular attention has been devoted to the NR2B subunit which seems to be activated in the spinal dorsal horn in close correlation with peripheral and central neuropathic pain (Guo et al., 2002; Abe et al., 2005; Pezet et al., 2008; Daulhac et al., 2011; Kim et al., 2012; Morel et al., 2013).
Beyond kinases activation, other post-translational and transcriptional modifications occur, notably Kv4.2 inactivation leading to an increase in membrane excitability (Hu et al., 2006, 2007) and induction of genes coding for proteins involved in neuronal sensitization, especially NK1 (substance P receptor), TrkB and COX2 (Ji et al., 2009). Although NMDA receptor activation is predominant in central sensitization, the implication of AMPA receptors and voltage-dependent calcium channels seems also of importance in this phenomenon (Blackburn-Munro et al., 2004; Garry and Fleetwood-Walker, 2004; Gwak et al., 2011).

V.2.d.2 Neurotrophins (Fig. 12)

Neurotrophins, namely NGF (nerve growth factor), BDNF, NT3 (Neurotrophin 3) and NT 4/5 (Neurotrophins 4/5), are well known for their role in neuronal survival and differentiation during development. In addition, they have important functions in adults. Neurotrophins are first synthetized as a pre-pro-mature form which has to be cleaved by proteases to generate mature neurotrophins that are recognized by high affinity tyrosine kinase receptors (Trk, implicated in cell survival and synaptic plasticity), and the low affinity p75 receptor (implicated in apoptosis). All neurotrophins interact with the p75 receptor, while NGF interacts specifically with TrkA, BDNF and NT4/5 with TrkB, and NT3 with TrkC (Pezet and McMahon, 2006).

Figure 12: Neurotrophins and their respective receptors (from Pezet and McMahon, 2006)

NGF

NGF is a peripheral mediator of pain, specifically expressed in chronic inflammatory and neuropathic pain states (Pezet and McMahon, 2006). Subcutaneous NGF administration induces hyperalgesia (Lewin et al., 1993; Joseph and Levine, 2010). In neuropathic pain states, NGF is produced and mostly released by Schwann cells and macrophages. NGF-induced neuronal sensitization is first mediated by TRPV1 phosphorylation which results in a marked decrease in activation threshold of this ionotropic receptor (Zhuang et al., 2005). Then, delayed actions of NGF consist of upregulating the expression of several neuronal sensitization factors including neuropeptides (SP, CGRP, BDNF) and cationic channels (TRPV1, P2X3, Nav 1.3, Nav1.8…) (Pezet and McMahon, 2006).

NT3

In contrast to the other neurotrophins, NT3 reduces hyperalgesia in neuropathic and inflammatory pain (Watanabe et al., 2000; Intondi et al., 2008). Its anti-hyperalgesic effect might be due to its inhibitory influence on the expression of voltage-dependent Na^+ channels and TRPV1 receptors (Wilson-Gerwing et al., 2005, 2008).

NT4/5

NT4/5 interacts with the same receptor as BDNF, and its effects are identical to those of the latter neurotrophic factor which are described in detail in the following paragraph.

BDNF

The neurotrophic factor BDNF is essentially known for its role in neuron survival, maturation and differentiation. Notably, BDNF seems to be necessary for the survival of sensory neurons during development (Hellard et al., 2004; Sedy et al., 2004). Furthermore, BDNF overexpression seems to be implicated in the initiation of inflammatory pain and peripheral cephalic and extracephalic neuropathic pain (Zhao et al 2006; Coull et al., 2005; Yajima et al., 2005, Constandil et al., 2012). However, some studies also showed that BDNF downregulation can be deleterious as well, notably in sensory neurons where this change seems to be correlated with thermal hypersensitivity (Lever et al., 2003). Indeed, after spinal cord injury, BDNF deficiency contributes to apoptosis of oligodendrocytes notably (Koda et al., 2002). Here, I will review the different signaling pathways which are - or might be - involved in the positive/negative modulation of pain by BDNF.

- Regulation of NMDA receptors (Fig. 13)

BDNF-mediated regulation of glutamatergic neurotransmission can occur at pre- and post-synaptic sites. At pre-synaptic sites, it has been shown in spinal cord slices from neonatal rats that BDNF can increase mEPSC frequency in lamina II neurons (Merighi et al., 2007). At post synaptic sites, a correlation between BDNF-induced neuronal hyperexcitability and NMDA receptor activation has been reported by Groth and Aanonsen (2002). Accordingly, these authors showed that the hyperalgesia induced by intrathecal BDNF could be dose-dependently reduced by administration of the NMDA receptor antagonist D(-)-2-amino-5-phosphonovaleric acid (D-APV).

BDNF increases NMDA receptor activation -at least in part- through an upregulation of the NR1 subunit possibly mediated by Fosb/ΔFosb in spino-thalamic neurons (Slack et al., 2004, 2005; McClung & Nestler, 2003). This upregulation is sustained by ERK, since a MEK inhibitor could reduce BDNF-induced NR1 up-regulation (Slack et al., 2004).

BDNF enhancement of NMDA-receptor-mediated glutamatergic neurotransmission is also sustained through phosphorylation-mediated activation of NR2B (Crozier et al., 1999; Carreno et al., 2011). Indeed, administration of the NR2B antagonist Ro 25-6981 completely blocks BDNF-induced allodynia (Geng et al., 2010). NR2B phosphorylation is, at least in part, mediated by Fyn kinases (Fig. 13). When BDNF binds to TrkB receptor, the resulting autophosphorylation of TrkB promotes its interaction with fyn kinases which then rapidly phosphorylates the NR2B subunit (Xu et al., 2006).

- Regulation of AMPA receptors (Fig. 13)

Activation of AMPA receptors sustains glutamatergic neurotransmission, thereby contributing to neuropathic and inflammatory pain (Katano et al., 2011; Kopach et al., 2012). Activation of AMPA receptors is driven by BDNF through two major pathways. First, BDNF can phosphorylate, via the activation of NMDA receptors, the GluR1 subunit of AMPA receptor thereby enhancing neuronal hyperexcitability (Wu et al., 2004). BDNF can also induce the phosphorylation and trafficking to the synapses of GluR1 subunits by activation of the CamKII and CREB (Fortin et al., 2012; Middei et al., 2013).

- Regulation of ion channels (Fig. 13)

BDNF regulates the expression of several ion channels in small and medium DRG cells expressing TRPV1 and BDNF itself. Notably, the tetrodotoxin-resistant Na(V) 1.9 channel, which is implicated in inflammatory and diabetic neuropathic pain (Craner et al., 2002; Amaya et al., 2006; Smith & Momin, 2008), has been shown to be mandatory for BDNF-induced depolarization (Blum et al., 2002). BDNF/TrkB-mediated increased neuronal excitability also seems to involve downregulation of voltage-dependent potassium channels Kv -1.2, -1.4 and -4.2 and the Ca^{2+} -activated K^{+} (BK) channels. Downregulation of these channels that increases the probability to trigger action potentials has been shown to be correlated with neuropathic pain (Park et al., 2003; Cao et al., 2010, 2012; Pollema-Mays et al., 2013).

- Regulation of sensory receptors (Fig. 13)

In addition to ion channels in sensory ganglia, BDNF can also regulate the expression and activation of sensory receptors. Indeed, in vitro, BDNF can upregulate the expression of TRPV1 and increase the activation of TRPA1 by chemicals in sensory neurons supporting the idea that it may play a role in thermal hyperalgesia and inflammatory pain (Ciobanu et al., 2009). ASIC channels are also regulated by BDNF signaling. The neurotrophic factor is essential for the expression of ASIC2 channels which detect low pH but are also important for normal mechanical transduction (McIlwrath et al., 2005). More recently, the role of ASIC1A channel in neuropathic pain has been highlighted by the demonstration that it can contribute to BDNF-induced hyperalgesia. According to Duan et al. (2012), the PI3K-AKT pathway is involved in BDNF-induced increase of ASIC1 current via phosphorylation of the channel (Fig. 13).

- Regulation of GABAergic neurotransmission

The regulation of GABA neurotransmission, and notably GABA receptors, by BDNF seems to be likely since, after chronic constriction injury to the sciatic nerve, downregulation of mRNA encoding GABA(A) receptors occurs almost exclusively in BDNF expressing small and medium DRG neurons (Obata et al., 2003). On the one hand, Pezet et al. (2002a) showed that intrathecal injection of BDNF can trigger a transient release of GABA in the spinal cord and concomitantly

reduce spinal nerve ligation-induced hypersensitivity to thermal and mechanical stimuli (Lever et al., 2003). These observations led to the conclusion that a reduction of BDNF availability occurs in neuropathic pain and causes an impairment of GABAergic neurotransmission in dorsal horn, thereby contributing to hyperexcitability and central sensitization.On the other hand, BDNF has also been demonstrated to reduce inhibitory currents by downregulating GABA A receptor expression in the hypothalamus and the hippocampus (Brunig et al., 2001; Jovanovic et al., 2004; Hewitt & Bains, 2006). At the spinal level, the evoked release of GABA and glycine as well as eIPSC in lamina II neurons are reduced in the presence of BDNF (Bardoni et al., 2007). Those two oppposite effects of BDNF on inhibitory currents make its implication in the modulation of inhibitory transmission difficult to assess. In reality, it seems that BDNF can transiently increase eIPSC, and causes secondarily long term depression of these currrents (Jovanovic et al., 2004).

Moreover, BDNF regulates GABAergic neurotransmission by reducing the plasma membrane addressing of K^+/Cl^- co-transporter 2 (KCC2) expressed in spinal nociceptive neurons. This transporter is implicated in Cl^- homeostasis by ensuring the anion exit from the cell. The decrease in membrane KCC2 density induced by TrkB receptor activation leads to an elevation of intracellular $[Cl^-]$ and reversal of the $[Cl^-]$ ext / $[Cl^-]$ int gradient. As a consequence, GABA A receptor activation no longer induces Cl^- influx but triggers a depolarizing exit of the anion (Price et al., 2009; Boulenguez et al., 2010; Hasbargen et al., 2010). This phenomenon occurs notably through BDNF produced by activated microglia after neural lesion (Coull et al., 2005; Boulenguez et al., 2010).

- Modulation of noradrenergic sprouting

The relieving effects of gabapentin and antidepressants rely on the release of noradrenaline and activation of α2 and/or β2 noradrenergic receptors (Yalcin et al., 2009a, 2009b; Bohren et al., 2013). BDNF seems necessary for the effect of these drugs affecting noradrenaline pathways through its stimulatory effect on noradrenergic sprouting following nerve injury. Indeed, intrathecal injection of BDNF is sufficient to induce noradrenergic sprouting (Hayashida et al., 2008). This effect of BDNF is associated with an up-regulation of tyrosine hydroxylase transcription through BDNF-mediated activation of the ERK/CREB pathway (Fukuchi et al., 2010). Interestingly, both TrkB and p75 receptors are implicated in BDNF-induced noradrenergic sprouting (Paqueron et al., 2001; Hayashida & Eisenach, 2011).

- Modulation of axonal sprouting

Long term potentiation (LTP) is mediated not only by an enhancement of the frequency and amplitude of excitatory currents, but also by modifications of the cytoskeleton and formation of new dendritic spines. One of the early immediate genes activated by BDNF is ARC 1 (Associated Regulated Cytoskeleton 1). Its expression is directly correlated to LTP initiation and is essential for the maintenance of newly formed synapses (Bramham, 2008).
Another protein upregulated by BDNF is Rac-1 (Lai et al., 2012). Its upregulation is mediated by cdk5 which is itself upregulated by Fosb/ΔFosb (McClung and Nestler, 2003). Rac-1 plays a key role in the formation of new dendritic spines in the spinal cord notably after spinal cord injury and peripheral nerve injury, and contributes to neuropathic pain (Erschbamer et al., 2005; Tan et al., 2008; 2011).
GAP-43, another protein which also regulates axonal sprouting after nerve lesion, can also be upregulated by BDNF (Coggeshall et al., 1991; Geremia et al., 2010; Wu et al., 2013a).

- Pain modulation by p75 and TrkB T1

Most of the studies on the effects of BDNF on pain are assuming that they rely on TrkB receptor only. However, BDNF can also bind, but with a low affinity, to p75 receptor and to the truncated forms of TrkB, TrkB T1 and T2 with an affinity equal to that for TrkB.
Signaling pathways downstream of these other receptors are quite different from those activated through TrkB, since, for example, p75 promotes apoptosis rather than survival and TrkB.T1 does not possess the intrinsic kinase activity of full length TrkB. As a matter of fact, truncated TrkB receptors should not be occulted because TrkB antagonists such as K-252a could also bind to them. On the other hand, it is known that p75 can participate in neuropathic and inflammatory pain notably by regulating CGRP expression in DRG (Obata et al., 2006a; Watanabe et al., 2008; Fukui et al., 2010).
Eventhough TrkB.T1 deletion does not change basal thermal and mechanical thresholds, it reduces inflammation and antiretroviral drug-induced pain (Renn et al., 2009). On the other hand TrkB. T1 expression is enhanced in DRG of rats with HIV-induced neuropathic pain (Maratou et al., 2009). Actually, the role of TrkB.T1 in central neuropathic pain has been recently demonstrated in a model of spinal cord contusion. Indeed, deletion of TrkB T1 results in a decrease in below-level neuropathic pain, associated with down regulated Iba-1 and GFAP expression (Wu et al., 2013b).

- BDNF autoinduction

The long lasting changes induced by BDNF are probably not only due to stable synaptic changes but may also involve BDNF autoinduction. Indeed, such an autoinduction has already been demonstrated in vitro (Yasuda et al., 2007) as well as in vivo in the hippocampus (Wibrand et al.,

2006). In DRG neurons, BDNF autoinduction seems to depend on CREB activation (Morioka et al., 2013).

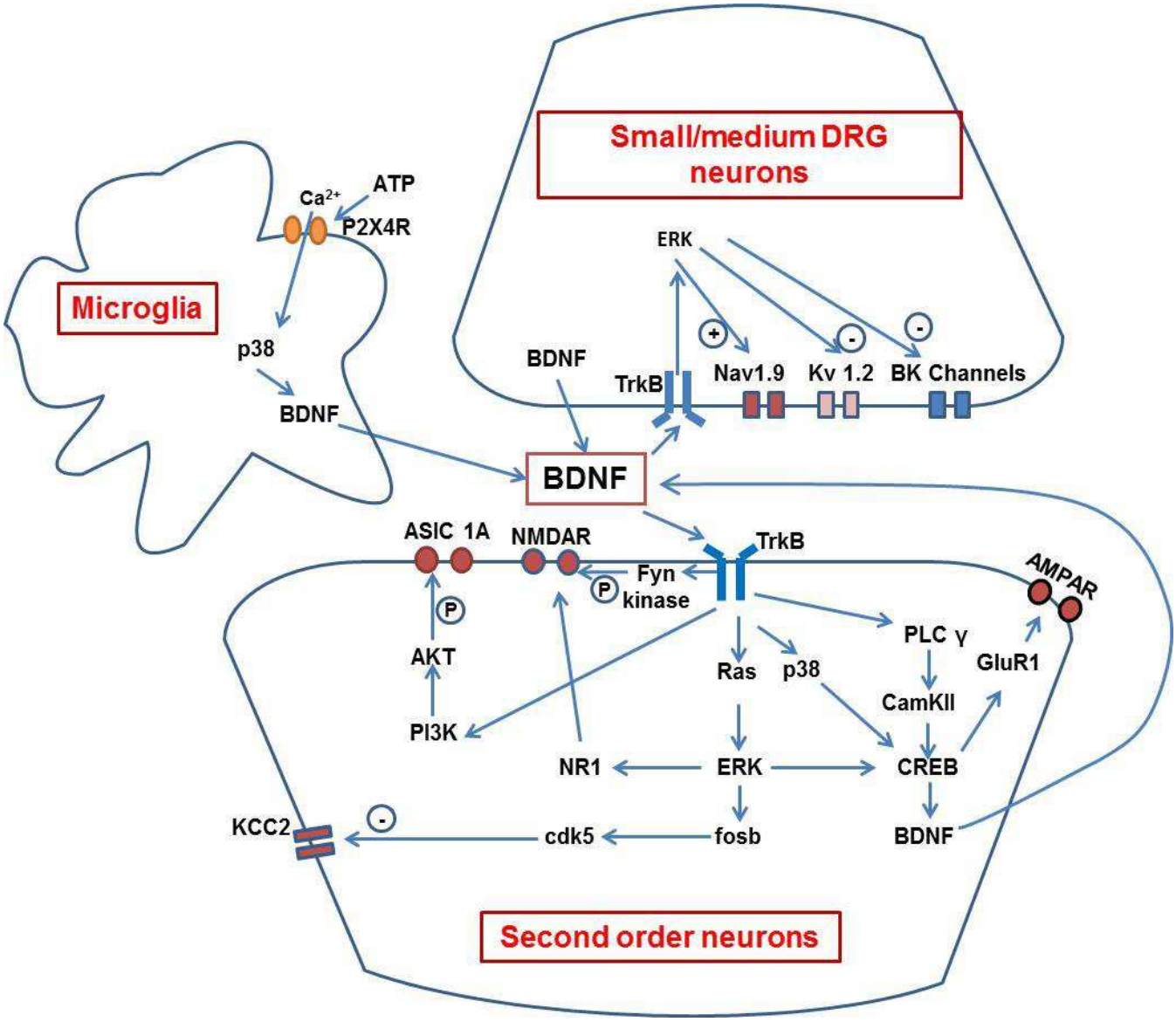

Figure 13: BDNF signaling pathways related to neuropathic pain. BDNF released from microglia and small/medium DRG neurons binds to TrkB receptors in the latter neurons and onand second order neurons in the dorsal horn of the spinal cord. The resulting activation of TrkB signalization induces marked functional changes in ion channels in DRG neurons. In second order neurons, the resulting activation of BDNF-induced TrkB activation triggers sensitization mostly via the activation of AMPA/NMDA receptors, ASIC1A channels and downregulation of KCC2 activity(from, Blum et al., 2002; McClung and Nestler 2003; Park et al., 2003; Slack et al., 2004 ; Xu et al., 2006; Trang et al., 2009 ; Cao et al., 2012; Duan et al.,

2012; Fortin et al., 2012 ; Huang et al., 2012; Middei et al., 2013 ; Pollema-Mays et al., 2013).

V.2.d.3 Long term potentiation

Memory and pain share common mechanisms and are clearly intricated. Indeed, how can a pain last for days, months, even years, after the primary insult if it has not been recorded somewhere in the spinal cord or in the brain (Ji et al., 2003) ? An important mechanism of memory involves LTP which can be induced by BDNF (see p.60). However, LTP is not only associated with memory but can also be activated by various noxious stimuli (Randić et al., 1993; Sandkühler and Liu, 1998; Rygh et al., 1999). This synaptic sensitization phenomenon depends principally on CaMKII and relies on glial cells (Ikeda and Murase, 2004; Pedersen et al., 2005). It can occur in spinal cord as well as in supraspinal areas such as the anterior cingulate cortex (Descalzi et al., 2009; Toyoda et al., 2009). LTP consists of the increase of synaptic strength between two neurites, which can last for days. It has two phases. Early phase corresponds to the phosphorylation of ionotropic glutamate receptors via kinases such as PKC, PKA or CamKII, which produces increases in NMDA and AMPA currents. The transition from early to late phase of LTP needs gene expression, protein synthesis and actin polymerization (Impey et al., 1998; Tanaka et al., 2008). The latter event, which occurs through the kinase Rac-1, is required for the maturation or the de novo formation of dendritic spines (Fischer et al., 1998).These spines allow an increased expression of AMPA and NMDA receptors with resulting increased magnitude and shorter latency of evoked potentials (Lüscher et al., 2000; Calabrese et al., 2006). A large body of evidence showed that dendritic spines are affected by various injuries or disease insults. In particular, it has been reported that the kinase Rac-1 a is required for the motility and stability of dendritic spines and promotes the clustering of AMPA receptors (Tashiro and Yuste, 2004) and therefore LTP. Rac-1 is upregulated at-, below- and above- the lesion from the 4^{th} day to 3 months after spinal cord injury and is implicated in central as well as peripheral neuropathic pain. Rac-1 blockade by NSC23766 reduces both dendritic spines formation and neuropathic pain through a mechanism totally independent of microglia activation (Erschbamer et al., 2005; Tan et al., 2008, 2011).

V.3. Epigenetic mechanisms in pain

In the sections above, we mentioned several processes, inflammatory, cellular, molecular that regulate central and peripheral neuropathic pain. The term epigenetics refers to processes that lead to stable and/or heritable changes in gene function without any concomitant DNA sequence changes. These processes include acetylation, methylation, phosphorylation of either histones or DNA. Generally, DNA methylation is associated with restriction of transcription, whereas acetylation of histones is associated with up-regulation of transcription (Denk and McMahon

2012). Developments in this new field have been particularly intense for the last years with the introduction of new tools to assess histones states with immunohistochemistry or CHIP (chromatin immunoprecipitation). Interestingly, epigenetics has recently emerged in the field of neurosciences especially about long term alterations associated with neuropathic pain in rodents models. Notably, BDNF up-regulation in DRG after peripheral nerve injury has been found to involve acetylation of H3 and H4 histones (Uchida et al., 2013). Moreover it has been shown that intrathecal administration of histones de-acetylases (HDAC) inhibitors prior to a neural injury could reduce by half the lesion-evoked neuropathic pain (Denk et al., 2013). Supraspinal areas such as the prefrontal cortex also revealed an up-regulation in DNA methylation after peripheral nerve injury (Tajerian et al., 2013). Clearly this field of research is especially promising for both a better knowledge of physiopathological mechanisms underlying neuropathic pain and the development of innovative therapies. Indeed, our perspective isto set up appropriate procedures to investigate the epigenetic mechanisms that account for the long term physiopathological alterations associated with the two neuropathic pain models that I studied for my PhD thesis.

OBJECTIFS DE LA THESE

Les douleurs neuropathiques se caractérisent par leur résistance aux traitements antalgiques conventionnels. Les médicaments qui sont utilisés aujourd'hui (antidépresseurs, anticonvulsivants) sont souvent peu efficaces et ont des effets secondaires qui peuvent être mal supportés. En réalité, il s'agit de composés dont l'action thérapeutique ne repose que sur des données empiriques. Une condition pour le développement de stratégies thérapeutiques innovantes, à la fois rationnelles et ciblées, est une meilleure connaissance des mécanismes physiopathologiques qui sous-tendent les douleurs neuropathiques. A cette fin, des modèles animaux pertinents, reflétant la pathologie chez l'homme et dont la mise en œuvre est aisément reproductible d'un laboratoire à un autre, sont nécessaires. Les modèles que nous avons étudiés jusqu'alors au laboratoire, à savoir la ligature du nerf sciatique/infraorbitaire, visent à remplir ces critères. Cependant, ces modèles ont deux défauts majeurs : (1) les lésions n'affectant que des nerfs périphériques, ils ne peuvent contribuer à l'acquisition de connaissances spécifiques sur les douleurs neuropathiques causées par une lésion du système nerveux central; (2) L'intensité des symptômes douloureux (hyperalgésie, allodynie) est variable d'un animal à l'autre, et une certaine proportion des animaux opérés ne développent pas de douleurs neuropathiques du fait de l'absence de critères objectifs pour le contrôle de la compression du nerf par la ligature.
Ces limites nous ont conduit à développer deux nouveaux modèles de douleurs neuropathiques pour tenter d'y remédier : d'une part, la section de la moelle épinière pour l'étude spécifique des douleurs neuropathiques d'origine centrale, et d'autre part, l'injection intrathécale de BDNF, pour une plus grande reproductibilité des symptômes douloureux que ceux induits par une intervention chirurgicale.
La première partie de ma thèse a donc été consacrée à l'étude des conséquences d'une section complète de la moelle épinière au niveau thoracique (T8-T9) chez le rat. Ce modèle a été choisi du fait de sa grande reproductibilité interindividuelle en termes de lésion, au contraire des modèles généralement utilisés consistant en la compression ou la contusion de la moelle épinière, souvent très variable d'un animal à l'autre. Afin de valider ce modèle de douleur centrale, nous avons tout d'abord évalué, avec le test des filaments de von Frey, l'évolution de la sensibilité mécanique du rat au cours du temps après l'opération. Sur l'ensemble du territoire corporel testé, les rats ont manifesté une hypersensibilité au niveau des pattes postérieures et une très forte allodynie au niveau du territoire cutané autour de la lésion. Cette allodynie est corrélée à une très forte induction des cytokines pro-inflammatoires ainsi qu'à une activation des cellules astrocytaires et microgliales à la fois dans la moelle épinière et dans les ganglions rachidiens

dans les segments thoraciques. Au plan pharmacologique, nous avons montré que l'allodynie générée par la section médullaire peut être réduite par l'administration aiguë de composés dont l'efficacité anti-allodynique est avérée chez l'homme médullo-lésé, en particulier la kétamine, le baclofène ou des opiacés. La caractérisation du modèle de section complète de la moelle au niveau thoracique que nous avons réalisée ouvre de nouvelles perspectives de recherche à la fois sur les mécanismes physiopathologiques sous-tendant les douleurs neuropathiques d'origine centrale et sur les voies de signalisation impliquées dans l'action anti-allodynique d'approches thérapeutiques validées ou potentielles.

La seconde partie de ma thèse a consisté à valider l'injection intrathécale unique de BDNF comme modèle de douleur neuropathique chez le rat. Comme souligné dans les rappels bibliographiques, l'implication du BDNF dans la douleur neuropathique est bien établie dans la littérature, de même que sa capacité à induire des symptômes tels que l'allodynie et l'hyperalgésie lorsqu'il est injecté par voie intrathécale. L'intérêt de ce modèle réside dans le fait qu'il ne nécessite aucune chirurgie et que l'allodynie et l'hyperalgésie mécaniques induites par le BDNF sont à la fois parfaitement reproductibles d'un rat à un autre, de relativement longue durée et d'une intensité équivalente à celle observée suite à la ligature d'un nerf périphérique. Notre travail a consisté à approfondir l'étude de ce modèle par la mise en œuvre de protocoles pharmacologiques et d'approches immunohistochimiques et moléculaires pour en préciser les caractéristiques physiopathologiques. Nous avons montré qu'une seule injection intrathécale d'une dose infra-nanomolaire de BDNF (3 ng) est suffisante pour induire une allodynie et une hyperalgésie de longue durée chez le rat. Des composés tels que la prégabaline et la morphine se sont montrés efficaces pour réduire ces symptômes de douleur neuropathique, validant ainsi l'homologie de notre modèle avec la pathologie chez l'homme en termes de réponses pharmacologiques. Par ailleurs, nous avons exploré, à la fois au plan cellulaire et moléculaire, les mécanismes d'induction et de maintien de l'allodynie et de l'hyperalgésie, et montré que l'injection intrathécale de BDNF n'entraine pas d'activation microgliale ni de neuroinflammation. Ce modèle, dont la mise en œuvre est beaucoup plus simple et reproductible que la réalisation d'une lésion chirurgicale, pourrait donc s'avérer d'un grand intérêt pour l'étude des voies de signalisation en aval de ces phénomènes dans les processus physiopathologiques sous-tendant les douleurs neuropathiques.

MATERIELS ET METHODES

I. ANIMAUX

Toutes les procédures impliquant les animaux ont été conduites en conformité avec les recommandations du Ministère de l'Agriculture et de la Forêt pour l'expérimentation animale. (Directive 87-848, du 19 Octobre 1987 ; Service vétérinaire de la santé et de la protection animale, autorisations d'expérimenter n° 006228 : Bourgoin S ; n° 75116 : Hamon M ; n° 00482 : Kayser V; n° A752128 : M'Dahoma S.)

De plus, les règles d'éthique de l'*International Association for the Study of Pain* (I.A.S.P ; Zimmermann, 1983) ont été scrupuleusement suivies (nombre minimum d'animaux utilisés, conditions optimales de stabulation, euthanasie immédiatement après la fin des expériences). Toutes les expériences ont été faites en accord avec les instructions du Ministère de l'Agriculture et de la Forêt concernant la recherche en neurosciences sur les animaux. Les expériences ont été réalisées sur des rats mâles Sprague-Dawley du Centre d'Elevage R. Janvier (53940, Le Genest St Isle) ou du centre d'élevage Charles River (69592, L'Arbresle). Les rats sont stabulés dans une animalerie agréée dont la température est fixée à 21±1°C tout au long du cycle jour (7 h-19 h)/nuit, avec la nourriture et la boisson *ad libitum* et une acclimatation d'une semaine dans l'animalerie avant le début des expériences.

II. MODELES DE DOULEUR NEUROPATHIQUE

II.1. Section de la moelle épinière au niveau thoracique

La section de la moelle épinière a été faite selon le protocole de Antri et al. (2003). Les animaux sont profondément anesthésiés avec de l'isoflurane à 5% pour l'induction et à 3% pour l'intervention chirurgicale. Après incision de la peau, les muscles paravertébraux sont écartés de chaque côté de la colonne vertébrale et la vertèbre T8 est ouverte (laminectomie). Une anesthésie locale des tissus est réalisée par refroidissement au cryoflurane (Promedica, France) quelques secondes avant la section complète de la moelle qui est pratiquée au niveau des segments T8-T9 avec des ciseaux ophtalmiques (Figure 14). L'insertion d'une mousse de gel hémostatique stérile (Surgicel; Ethicon, Somerville, NJ, USA) au niveau de la section permet de s'assurer qu'elle est bien complète et d'arrêter un éventuel saignement. Ensuite les muscles sont suturés et l'incision de la peau est refermée à l'aide d'agrafes chirurgicales. Les animaux témoins (« sham ») subissent l'ensemble de l'intervention chirurgicale à l'exception de la section de la moelle épinière. Après la chirurgie, les rats sectionnés et les « sham » sont répartis à deux par cage afin

de limiter les risques de blessure par les autres animaux. Au cours des sept jours suivants, tous les rats sont traités par des antibiotiques (oxacillin, Bristopen ®, Bristol Myers Squibb S.P.A Italie, 0.3 mg/100 g s.c ; gentamicin, Panpharma, France, 0.2 mg/100 g s.c.) afin d'éviter d'éventuelles infections (notamment par des staphylocoques). Les animaux sont aussi massés quotidiennement au niveau de la vessie pendant approximativement 10 jours, le temps que le réflexe de miction revienne.

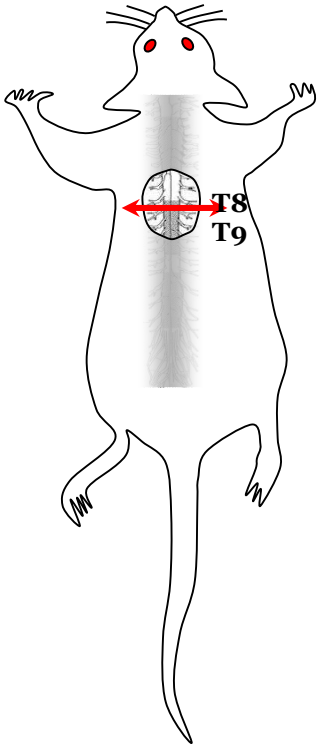

Figure 14 : Transection de la moelle thoracique au niveau T8-T9 chez le rat.

II.2. Ligature unilatérale du nerf sciatique (rats CCI-SN)

Les animaux sont anesthésiés au pentobarbital (50 mg/kg, i.p.), et le tronc commun du nerf sciatique de la cuisse droite est dégagé après incision du biceps fémoral. Quatre ligatures lâches (réalisées à l'aide d'un fil de soie, 5.0) sont ensuite placées autour du nerf sciatique (*Figure 15*), avec un espace d'environ 1 millimètre entre elles. Puis les ligatures sont serrées de manière à ralentir la circulation épineurale sans la bloquer complètement (Bennett et Xie, 1988). La ligature induit une constriction ischémique progressive

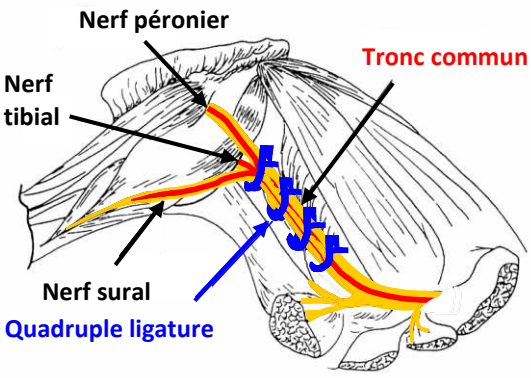

Figure 15 : Ligatures du nerf sciatique

du nerf et une inflammation. Cette lésion conduit à l'apparition d'une allodynie et d'une hyperalgésie au niveau de la patte au nerf ligaturé, dont les intensités sont maximales à partir de deux semaines après la pose des ligatures (Latrémolière et al., 2008). Des animaux *sham*, qui subissent la même intervention chirurgicale mais sans la pose de ligatures, sont opérés en même temps que les animaux CCI-SN.

II.3. Injection intrathécale de BDNF (rats « BDNF i.t.)

Les animaux sont légèrement anesthésiés à l'isoflurane 3%. L'injection de 12 µl de sérum physiologique (0.9% NaCl) ou de 3 ng de BDNF dans le même volume est réalisée à l'aide d'une seringue Hamilton, dans l'espace sous-arachnoïdien entre les vertèbres L5 et L6. Le mouvement réflexe de la queue du rat confirme la bonne localisation de l'aiguille pour l'injection. Dès 4-5 jours après l'injection i.t. de BDNF, apparaissent une allodynie et une hyperalgie au niveau des pattes postérieures, dont les intensités sont maximales 10 jours après l'injection.

III. TRAITEMENTS PHARMACOLOGIQUES

Les agents pharmacologiques utilisés pour les différents projets sont les suivants (Tableaux 5 et 6):

la gabapentine (acide 2-[1-(aminométhyl)cyclohexyl]acétique ; Sequoïa, Pangbourne, G-B.) ; la prégabaline [acide (*S*)-3-(aminométhyl)-5-methylhexanoique ; Sequoïa, Pangbourne, G-B.), le baclofène [acide (*S*)-3-(aminométhyl)-5-methylhexanoique ; Research Biochemical Inc), l'amitriptyline [3-(10,11-dihydro-5*H*-dibenzo[a,d] cycloheptene-5-ylidene)-*N*, *N*-dimethyl-1-propanamine : Sigma Aldrich)], la ketamine [(*RS*)-2-(2-chlorophenyl)-2-(methylamino) cyclohexanone ; Sigma Aldrich], le 8-OH-DPAT [(±)-8 hydroxy-2-dipropylamino-tetraline ; Sigma Aldrich] , la cyclotraxine B (Bio S&T, Montreal, Canada), le naratriptan [(N-methyl-3-(1-methyl-4-piperidinyl)-1H-indole-5-ethane sulphonamide ; Glaxo-Wellcome)], le tapentadol [(3-[(1R, 2R)-3-(dimethylamino)-1-ethyl-2-methylpropyl]-phénol ; Grünenthal, Aachen, Allemagne], la morphine [7,8-didéhydro-4,5-époxy-17-méthylmorphinan-3,6-diol : Pharmacie Centrale des Hôpitaux de Paris], l'agomélatine {N-[2-(7-methoxy- 1- naphtyl) éthyl] acétamide, Servier}, la duloxetine [(3S)-N-methyl-3-naphthalene-1-yloxy-3-thiophene-2-ylpropane-1-amine ; Sequoïa], l'ondansetron [(RS)-2,3-dihydro-9-méthyl- 3-[(2-méthylimidazol-1-yl)méthyl] carbazol-4(1H)-one ; Glaxo-Wellcome] et le clonazepam [7-nitro-5-(2-chlorophényl)-2,3-

dihydro-1H-benzo[f]-1,4-diazépin-2-one : Roche].

Les modes d'administration et les doses ont été choisis en fonction d'études antérieures. Tous les composés ont été dissous dans le sérum physiologique (NaCl :0,9%) à l'exception de la duloxétine et de l'agomélatine qui ont été suspendus dans de l'hydroxyethylcellulose (HEC) à 1% dans l'eau milliQ, du baclofène dissous dans un mélange DMSO :NaCl 0,9% (50 :50) et du clonazepam suspendu dans un mélange éthanol:eau (50 :50). Le volume injecté est de 1 ml/kg de poids corporel. Les animaux témoins reçoivent les véhicules respectifs par la même voie d'administration.

Traitements	Cibles	Doses
8-OH-DPAT	Agoniste des récepteurs 5-$HT_{1A/7}$	0,25 mg/kg s.c.
Amitriptyline	Antidépresseur tricyclique	10 mg/kg i.p.
Baclofène	Agoniste des récepteurs GABA-B	5, 10 mg/kg i.p.
Clonazepam	Benzodiazépine, agoniste du site BZD des récepteurs GABA-A	0,25, 2 mg/kg i.p.
Cyclotraxine B	Antagoniste des récepteurs TrkB	20 mg/kg i.p.
Gabapentine	Bloquant de la sous unité α2δ des canaux calcium voltage-dépendants	30, 100, 300 mg/kg i.p
Kétamine	Antagoniste des récepteurs NMDA	50 mg/kg i.p.
Morphine	Agoniste des récepteurs opioïdes	1, 3, 10 mg/kg s.c.
Naratriptan	Agoniste des récepteurs 5-$HT_{1B/1D}$	0,1 mg/kg s.c.
Ondansetron	Antagoniste des récepteurs 5-HT_3	20 µg i.t.
Prégabaline	Bloquant de la sous unité α2δ des canaux calcium voltage-dépendants	30 mg/kg i.p
Tapentadol	Agoniste des récepteurs opioïdes µ et inhibiteur de la recapture de noradrénaline	10, 20 mg/kg i.p

Tableau 5 : Agents pharmacologiques administrés chez les rats médullo-lésés. Les traitements ont été effectués en aigu 30 jours après la transection de moelle épinière.

Traitements	Cibles	Doses
Agomélatine	Agoniste des récepteurs MT1 et MT2 et antagoniste des récepteurs 5-HT_{2B} et 5-HT_{2C}	45 mg/kg i.p
Amitriptyline	Antidépresseur tricyclique	10 mg/kg i.p.
Clonazepam	Benzodiazépine, agoniste du site BZD des récepteurs GABA-A	0,25, 2 mg/kg i.p.
Cyclotraxine B	Antagoniste des récepteurs TrkB	20 mg/kg
Duloxétine	Inhibiteur sélectif de la recapture de sérotonine et de noradrénaline	10 mg/kg i.p
Morphine	Agoniste des récepteurs opioïdes	3 mg/kg s.c.
Prégabaline	Bloquant de la sous unité α2δ des canaux voltage calcium-dépendants	30 mg/kg i.p
Tapentadol	Agoniste des récepteurs opioïdes μ et inhibiteur de la recapture de noradrénaline	10 mg/kg i.p

Tableau 6 : Agents pharmacologiques administrés chez les rats « BDNF i.t. » et les rats CCI-SN. Les traitements en aigu ont été effectués respectivement 10 ou 15 jours après l'injection intrathécale de BDNF ou la ligature du nerf sciatique.

IV. TESTS D'ALGESIMETRIE

Afin de quantifier les manifestations comportementales en réponse à des stimulations tactiles calibrées et d'évaluer les éventuels effets antalgiques/proalgiques des différents traitements, deux tests ont été utilisés : le test des filaments de von Frey pour évaluer l'allodynie et le test de Randall et Selitto pour l'hyperalgésie.

IV.1. Test des filaments de Von Frey

Ce test consiste en l'application d'une pression très localisée réalisée au moyen d'un filament de nylon au niveau d'un territoire cutané. Chaque filament de von Frey, fixé perpendiculairement à l'extrémité d'un manche en plastique, permet d'exercer une pression calibrée qui varie selon son diamètre. Nous l'avons utilisé pour mettre en évidence et mesurer l'allodynie mécanique à la suite de la section de moelle épinière, de la ligature unilatérale du nerf sciatique ou de l'injection intrathécale de BDNF.

IV.1.a. Au niveau des pattes postérieures et antérieures

Chaque rat est placé sur une grille métallique horizontale (maillage de 1 cm afin de laisser passer le filament) à 1 m au-dessus de la paillasse, sous une boîte en plexiglas transparent de dimensions 31 x 19,5 cm, h=14 cm (Fig. 16). Des filaments exerçant une force parfaitement calibrée de 4, 6, 8, 10, 12, 15, 26 ou 60 g sont appliqués les uns après les autres dans l'ordre croissant jusqu'à leur point de courbure. La procédure consiste en l'application de 3 stimulations, chacune espacée de 3 secondes, au niveau de la patte postérieure, puis 5 minutes plus tard, au niveau de la patte antérieure de l'animal. Un temps d'attente de 5 minutes est respecté entre deux séquences de stimulation. Pour chaque filament appliqué, on compte le nombre de réactions aversives (retrait de la patte) afin de déterminer le seuil d'intolérance de l'animal.
La pression maximale (« point limite ») dans ce test est exercée au moyen du filament de 60 g (ou 100 g chez les animaux médullo-lésés). Elle évite tout risque de lésion qui pourrait survenir pour des pressions plus élevées.

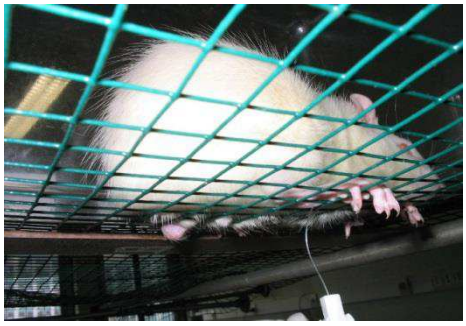

Figure 16 : Test des filaments de von Frey au niveau de la patte.

IV.1.b. Dans le territoire cutané au niveau de la transection spinale

Ce test a d'abord été systématiquement appliqué sur tout le territoire corporel (y compris au niveau céphalique) chez les animaux opérés. Il n'a ensuite été pratiqué qu'au voisinage de la cicatrice de l'intervention chirurgicale, seule région qui présente une importante allodynie pendant au moins deux mois après la transection spinale. Les rats sont placés individuellement dans une cage en plexiglas de dimension 42x24 cm h=15 cm. L'allodynie mécanique est évaluée avec une série de filaments de von Frey exerçant une pression de 0,008 g à 100 g. Chaque filament est appliqué sur le dos de l'animal autour de la lésion dans une zone de 6 cm^2, en commençant par le filament produisant la pression la plus faible (Fig. 17). Trois stimulations sont effectuées avec chaque filament toutes les cinq secondes. Si la zone testée n'est pas allodynique le filament est appliqué 1 minute plus tard dans une zone adjacente jusqu'à couvrir tout le territoire donnant une réponse, puis on passe au filament suivant. La pression minimale

pour déclencher une réaction nocifensive de type : ébrouement, tentative de morsure ou fuite est considérée comme étant « le seuil de pression » mécanique. La pression maximale (« point limite ») dans ce test est exercée au moyen du filament de 100 g. Elle évite tout risque de lésion qui pourrait survenir pour des pressions plus élevées. Avant opération, le filament de von Frey de 100 g est testé chez tous les animaux afin de vérifier qu'il ne provoque aucune réaction nocifensive. Les quelques rares animaux qui réagissent à l'application de ce filament sont immédiatement exclus de l'étude.

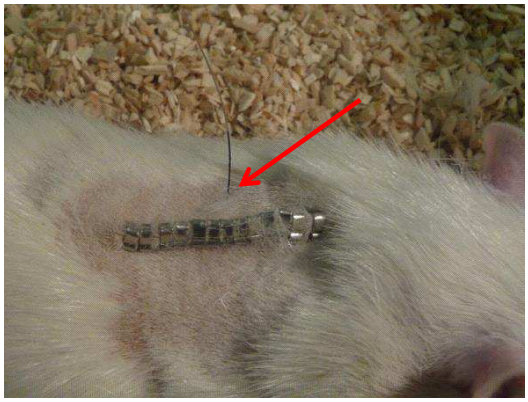

Figure 17 : Test des filaments de von Frey dans le territoire cutané au niveau de la transection spinale.

IV.2. Test de pression de la patte

Figure 18 : Test de pression de la patte

Le test de pression de la patte (Randall et Selitto, 1957) permet de mesurer l'hyperalgésie mécanique. Il consiste, à l'aide du stylet d'un stimulateur mécanique activé par l'expérimentateur (algésimètre Ugo Basile, 92370 Chaville), à exercer une pression croissante sur la face dorsale de la patte postérieure droite du rat (c'est-à-dire du côté ipsilatéral à la ligature du nerf sciatique, dans le cas des animaux CCI-SN). La pression est appliquée entre le 3ème et le 4ème métatarsien (territoire innervé par le nerf sciatique) (*Figure 18*). L'expérimentateur tient l'animal enveloppé dans un linge de façon à ne laisser émerger que la tête et les pattes postérieures et à limiter le stress de contention. Deux seuils de pression sont déterminés au cours de l'augmentation progressive de la pression appliquée: le premier correspond à la pression pour laquelle le rat essaie de retirer sa patte (seuil de retrait) et le second est la pression minimale déclenchant une plainte (seuil de vocalisation) lorsque la patte est maintenue sous le stylet au-delà de la pression-seuil de retrait. Le retrait de la patte est un mouvement réflexe qui implique un réseau neuronal spinal alors que la vocalisation

fait intervenir des structures supraspinales impliquées dans la transmission et l'intégration des messages nociceptifs (Le Bars et al., 2001).

IV.3. Conditions expérimentales

Tous les tests ont été effectués dans une salle dont la température était neutre (22-24°C) et l'éclairage le plus stable possible (éclairage artificiel, lumière naturelle tamisée). Le passage des tests s'est toujours déroulé entre 9 h et 16 h. Dans tous les cas, les animaux ont été placés dans la salle d'expérimentation au moins une heure avant le début du test, afin de les habituer au nouvel environnement.

V. RT-PCR SEMI QUANTITATIVE EN TEMPS REEL

Après sacrifice par décapitation, les ganglions des racines dorsales correspondant à T6, T7 et T8 (au-dessus de la section) et T9, T10 et T11 (au-dessous), et des segments de 5 mm de moelle épinière au-dessus et en dessous de la section, ainsi que les renflements lombaire et cervical sont prélevés chez les rats ayant subi une transection de la moelle épinière. Chez les animaux BDNF i.t., la moelle lombaire dorsale et les ganglions L4-L5-L6 sont prélevés tandis que chez les rats CCI-SN seule la moelle lombaire dorsale ipsilatérale et les ganglions associés (L4-L6) sont disséqués. Les tissus sont immédiatement congelés dans l'azote liquide puis conservés à -80°C. Les ARNm sont extraits à température ambiante à l'aide d'un kit (Macherey-Nagel, NucleoSpin® RNA II) comportant tous les tampons et réactifs nécessaires. Les tissus sont homogénéisés dans un tampon de lyse contenant 1% de β-mercaptoethanol. Le lysat est débarrassé des débris et clarifié par filtration sur colonne (centrifugation à 11.000 x g, 1 min). L'ajout d'éthanol (35% final) optimise les conditions de fixation de l'ARNm sur une 2ème colonne (Nucléospin® RNA II). Après dessalage, l'ADN génomique dans l'échantillon est éliminé sous l'action de la DNase, l'échantillon est soumis à différents lavages, et l'ARNm est élué avec de l'eau « RNase free ». Sa concentration est déterminée par spectrophotométrie (Nanodrop), et sa qualité vérifiée par électrophorèse sur gel d'agarose. Les échantillons sont ensuite conservés à -20°C.

Les ARNm de chaque échantillon sont soumis à la transcriptase inverse, qui génère une copie d'ADNc à partir de chaque brin d'ARNm, à l'aide d'un kit (High capacity cDNA Reverse Transcription kit, Applied Biosystems). La désactivation de l'enzyme est ensuite réalisée par chauffage à 85°C pendant 5 sec, immédiatement suivi d'un refroidissement à 0°C. Les

échantillons, analysés en triplicat, subissent une amplification par PCR semi quantitative réalisée à l'aide d'un appareil Applied Biosystems Prism 7300 (Applied Biosystems, Courtaboeuf, France) avec un kit TaqMan® Universal PCR Master Mix No AmpErase® UNG (Applied Biosystems) et des amorces spécifiques de gènes d'intérêt (fournies par Applied Biosystems). Les références de amorces utilisées sont les suivantes : ATF3 (assay ID Rn00563784_m1), GFAP (Rn01460868_m1), OX42 (Rn00709342_m1), IL6 (Rn00561420_m1), IL1β (Rn00580432_m1), BDNF exon IX (Rn02531967_s1), TLR4 (Rn00569848_m1), P2X4 (Rn00580949_m1), P2X7 (Rn00570451_m1), IL-10 (Rn00563409_m1) TNF-α (Rn00562055_m1), AIF-1 (Rn00574125_g1), NR2B (Rn00680474_m1), TrkB (Rn01441749_m1). Le gène de la glycéraldéhyde-3-phosphate déshydrogénase (GAPDH; Rn99999916_s1), enzyme ubiquitaire dont la concentration de l'ARNm ne varie dans aucune condition expérimentale connue, est utilisé comme référence. L'étape d'activation de la polymerase à 95°C est suivie de 40 cycles de 15 secondes à 95°C et 60 secondes à 60°C. Les niveaux d'ARNm spécifiques ont été calculés en normalisant par rapport au transcrit GAPDH en utilisant la méthode $2^{-\Delta\Delta Ct}$ (Schmittgen and Livak, 2008).

VI. IMMUNOHISTOCHIMIE

Les marquages immunohistochimiques ont été réalisés le plus souvent sur des coupes de moelle épinière provenant d'animaux lésés ou injectés en i.t. par le BDNF et soumis à aucun autre traitement.

Ce n'est que pour quelques séries expérimentales que les animaux ont été soumis à une stimulation mécanique répétée avant le sacrifice et la mise en œuvre des procédures d'immunomarquages.

VI.1. Stimulation mécanique de la patte postérieure

Afin de vérifier par une approche immunohistochimique qu'une stimulation mécanique allodynique entraîne bien une activation microgliale dans la corne dorsale de la moelle épinière chez des rats présentant une sensibilisation neuronale (à la suite de l'injection intrathécale de BDNF ; voir Résultats), nous avons soumis les animaux à une stimulation mécanique répétée avec une brosse au niveau de la patte postérieure. Plus précisément, une séquence de 5 secondes de frottements (depuis l'arrière jusqu'à l'avant de la face dorsale de la patte) et de 10 secondes d'arrêt a été répétée pendant 10 min. Les animaux ont été sacrifiés 24 h après le début des

stimulations pour la préparation des coupes de moelle dorsale destinées à l'immunomarquage de la forme activée (phosphorylée) de la MAP kinase p38. La sensation ressentie d'une telle stimulation appliquée sur une main correspond à un frottement soutenu mais non douloureux.

VI.2. Immunomarquage de protéines d'intérêt

Les animaux sont anesthésiés avec du pentobarbital (50 mg/kg, i.p.) et perfusés en intracardiaque avec du sérum physiologique hépariné (5000 UI/ml) pendant 8 minutes, afin d'éliminer le sang des tissus. Une 2ème perfusion est réalisée avec du tampon phosphate de sodium (0,005 M, pH 7,4) contenant du paraformaldéhyde à 4%, de l'acide picrique (0,1%) et du glutaraldéhyde (1%), pendant 25 minutes, pour fixer les tissus. Enfin, les animaux sont perfusés avec une solution de saccharose à 20%, pendant 5 minutes. Le renflement lombaire (L3-L6) de la moelle épinière est ensuite prélevé, et immergé dans la solution de saccharose pour un stockage à 4°C pendant 24 h. Pour le repérage anatomique, la partie de la moelle ventrale contralatérale à la patte au nerf ligaturé ou stimulée est marquée par une fente. Les segments de moelle sont conservés à -80°C dans du cryotech avant d'être sectionnés en coupes de 30 µm d'épaisseur à l'aide d'un cryostat, puis les coupes sont rincées dans du tampon phosphate salin (PBS, 0,02M) et conservées dans du PBS 0,02M contenant 0,2% d'azide de sodium.

Pour l'immunomarquage, les coupes sont rincées 5 fois avec du PBS 0,02M, puis incubées 10 min à température ambiante, dans du péroxyde d'hydrogène 30% dilué au 1/100^{e} dans du PBS-T (Triton X-100, 0.3%) afin d'inactiver la péroxydase endogène. Ensuite, les coupes sont rincées 5 fois 5 minutes dans le PBS 0,02M, avant d'être incubées toute une nuit à température ambiante dans une solution d'anticorps primaire diluée dans du PBS-T azide (Triton X-100, 0,3% ; azide 20%).

Les anticorps primaires qui ont été utilisés sont les suivants : anticorps anti-Iba1 (1:8000, Wako Chemical Inc), anticorps anti-P-p38 (1:3000, Cell Signaling Inc), anticorps anti-fosb (1 :1500, Santa Cruz), l'anticorps anti P-CREB (1:300, Cell Signaling Inc).

Les coupes sont ensuite rincées dans du PBS 0,02M, avant d'être incubées 1 h à température ambiante dans la solution d'anticorps secondaire biotinylé anti-lapin diluée au 1/1000 dans du PBS-T (Triton X-100, 0.3%) Après un nouveau rinçage dans le PBS 0,02M, elles sont incubées 1 h à température ambiante dans le complexe avidine-biotine dilué au 1/500 dans du PBS 0,02M. Puis les coupes sont de nouveau rincées dans le PBS 0,02M, et le marquage est révélé avec un « kit de substrat » de la péroxydase (3,3'-diaminobenzidine, Vector laboratories). Le temps de révélation est de 1 min 30 pour l'anticorps anti Iba1, 9 min pour l'anticorps anti-P-p38, 4 min 30

pour l'anticorps anti-fosb et 15 min pour l'anticorps anti P-CREB. Les coupes sont finalement rincées 5 fois dans le PBS 0,02M, et montées sur lames pour le traitement dans des bains d'éthanol et de xylène. Suite à ce traitement, une lamelle est collée sur chacune des lames avec de l'Eukitt.

Le comptage des cellules immunomarquées dans la substance grise est fait à l'aide du logiciel Mercator® dans les régions prédéfinies de la moelle épinière. Pour les comparaisons statistiques, des différents groupes de rats, nous avons pris soin de réaliser le comptage très précisément dans les mêmes zones des coupes. Des microphotographies ont été prises avec une caméra vidéo couleur CCD connectée au microscope (grossissement x20) pour l'envoi des signaux de sortie RGB (Red, Green, Blue) à un micro-ordinateur Macintosh. Des images dans différents plans de la même région ont été prises et digitalisées en niveaux de couleur 24 bit en utilisant le logiciel Openlab (Improvision, Coventry, G-B). Elles ont ensuite été fusionnées en une seule image en incorporant la valeur la plus sombre de chaque pixel correspondant à chaque plan focal pour les plans rouge, bleu et vert.

Les photos sont ensuite exportées vers Adobe-Photoshop (version 6.0) afin de rassembler les images adjacentes pour un montage à haute résolution et de grand angle. Puis la luminosité, le contraste et la taille de l'image sont ajustés.

VII. CALCULS STATISTIQUES

Les résultats sont exprimés sous la forme de la moyenne + E.S.M. (Erreur Standard à la Moyenne). Pour les données de RT-qPCR, et d'immunohistochimie, lorsque l'étude ne comportait que deux groupes d'animaux, un test de Student a été utilisé pour mettre en évidence d'éventuels effets significatifs. Pour les données de RT-qPCR contenant plus de deux groupes d'animaux (suivi temporel chez les animaux SCT), une analyse de variance à deux voies (two-way ANOVA) suivi d'un test de Bonferroni ont permis de comparer les données de chaque groupe par rapport à leurs contrôles. Concernant les données d'immunohistochimie, une analyse one-way ANOVA suivie d'un test de Newman-Keuls ont été effectués lorsque l'étude comportait 3 groupes ou plus. Pour les données pharmacologiques, les valeurs des groupes traités versus les contrôles sont comparées grâce à une analyse de variance à deux voies (two-way ANOVA) suivie, en cas de test positif, par un test de Bonferroni ou le cas échéant par une analyse one-way ANOVA suivie d'un test de Dunnett. Pour les AUC (aires sous la courbe), le test de Student a été utilisé. Dans tous les cas, le seuil de significativité a été fixé à $P < 0,05$.

RESULTATS

ARTICLE 1

Caractérisation comportementale, physiopathologique et pharmacologique de la douleur neuropathique centrale chez le rat ayant subi une transection de la moelle épinière

I - INTRODUCTION

Les lésions de la moelle épinière sont particulièrement invalidantes non seulement de par les déficits majeurs au plan moteur et neurovégétatif qu'elles engendrent mais aussi du fait de l'incidence des douleurs neuropathiques chez les patients médullo-lésés. Plus de 40 % des patients souffrent de douleurs neuropathiques sévères qui peuvent être localisées au niveau des dermatomes, au-dessus ou en dessous du site de la lésion (Donovan et al., 1982; Finnerup et al., 2001; Bryce et al., 2006, 2012). Ces douleurs sont, de plus, particulièrement réfractaires aux traitements antalgiques classiques. Il est donc indispensable de disposer de modèles animaux pertinents afin d'étudier la douleur neuropathique centrale, en élucider les mécanismes physiopathologiques et identifier de nouvelles cibles pour des traitements innovants, à la fois efficaces et bien tolérés.

De nombreux modèles animaux de lésion de moelle épinière (contusion, compression, ischémie…) ont été développés pour l'étude de la douleur neuropathique centrale (Nakae et al., 2011). Chacun de ces modèles présente des caractéristiques différentes en termes de localisation et de type de douleur. Cependant, ils ont tous le défaut majeur de conduire à des variations inter-individuelles importantes qui concernent notamment l'étendue et la sévérité de la lésion induite (Basso et al., 1996), l'intensité et la nature même des douleurs (Crown et al., 2006). Dans le but de disposer d'un modèle de douleur neuropathique centrale plus reproductible, nous avons étudié au niveau comportemental, physiopathologique et pharmacologique, les caractéristiques de la douleur neuropathique générée par une section complète de la moelle épinière au niveau T8-T9 chez le rat. L'évaluation de ce modèle SCT (« *Spinal Cord Transection* ») a, en premier lieu, consisté à mesurer les conséquences de la lésion sur la sensibilité mécanique à l'aide du test des filaments de von Frey appliqué à différents territoires corporels.

Dans un deuxième temps, nous avons recherché quels étaient les mécanismes physiopathologiques associés à l'allodynie mécanique consécutive à la lésion en analysant, par qRT-PCR, l'expression de gènes de la neuroinflammation au niveau de la moelle épinière et des ganglions des racines dorsales. Nous avons notamment précisé l'évolution temporelle de l'expression des marqueurs d'activation microgliale (OX-42) et astrocytaire (GFAP) dont l'induction avait déjà été mise en évidence dans d'autres modèles de lésion de moelle épinière

présentant des douleurs neuropathiques (Carlton et al., 2009). Comme l'activation des cellules gliales implique les récepteurs purinergiques P2X4 et P2X7 et le récepteur Toll-like de type TLR4, nous avons également quantifié leurs transcrits chez les rats médullo-lésés. Enfin, l'activation microgliale/astrocytaire entrainant la production et la libération de cytokines pro- et anti-inflammatoires (Kigerl et al., 2007 ; De Rivero Vaccari et al., 2012 ; Marcillo et al., 2012), les ARNm de IL-6, IL1β, TNFα et IL-10 ont été mesurés en parallèle à différents temps après la section de la moelle épinière.

Dans une troisième et dernière étape en vue de valider notre modèle sur le plan pharmacologique, nous avons recherché si l'allodynie consécutive à la section spinale pouvait être diminuée par les molécules utilisées pour soulager les patients médullo-lésés, voire d'autres composés potentiellement antalgiques.

II - RESULTATS

II.1 - Conséquences cliniques de la section complète de la moelle thoracique chez le rat SCT

Les problèmes de rétention urinaire et d'hématurie présents chez les animaux ayant subi une section totale de la moelle épinière ont pu être réglés en une semaine par le massage manuel quotidien au niveau de la vessie. Hormis cela, les rats SCT ont présenté, après la première semaine, un état général satisfaisant pendant les deux mois de l'étude, avec un pelage propre, une prise de poids normale, et nous n'avons jamais décelé le moindre signe d'autotomie.

II.2 - Développement d'une hypersensibilité au niveau des pattes postérieures

Après section de la moelle épinière, nous avons observé, chez les rats SCT par rapport aux rats « sham », une baisse du seuil de pression pour déclencher le retrait réflexe de la patte postérieure (test des filaments de von Frey) significative dès 18 jours après la section. Cette baisse s'accentue ensuite jusqu'à un seuil voisin de 10% de celui des rats « sham » au 50-60ème jour après la section de moelle.

II.3 - Développement de l'allodynie au niveau du territoire cutané autour de la lésion

Au niveau du territoire dorsal en arrière de la lésion, aucune réaction à l'application des filaments de von Frey n'a pu être détectée chez les rats SCT, même pour la pression testée la

plus forte : 100 g. Cependant, dans le territoire dorsal en avant de lésion, nous avons observé le développement d'une allodynie, qui atteint son maximum 30 jours après la lésion (réduction du seuil de réaction nocifensive d'environ 99% par rapport aux rats « sham »). L'étendue de ce territoire allodynique reste limitée puisqu'elle correspond à une surface cutanée d'environ 6 cm².

II.4 - Etudes pharmacologiques

Trente jours après l'opération, les rats médullo-lésés ont été traités en aigu avec différents agents pharmacologiques dans le but de mesurer leurs effets sur l'allodynie mécanique au niveau de la lésion. Parmi tous les composés testés, seuls la morphine, le tapentadol et la kétamine ont complètement supprimé l'allodynie au niveau de la lésion, de manière transitoire. Le baclofène a aussi réduit l'allodynie mais son effet est resté très modeste et de courte durée.

II.5 - Suivi de la réaction neuroinflammatoire par qRT-PCR dans les ganglions des racines dorsales et la moelle épinière chez le rat SCT

- *Les marqueurs de souffrance neuronale, ATF3, et d'activation gliale, OX-42 et GFAP*

Au niveau de la moelle épinière au-dessus et en dessous de la section, les ARNm d'ATF3 et d'OX-42 sont augmentés chez les rats médullo-lésés dès 2 jours après l'opération et le restent jusqu'au dernier jour de l'étude (60 j). L'ARNm de GFAP n'est respectivement augmenté qu'à partir de 4 jours et 21 jours après la section, au-dessus et en dessous de la section, et ceci (au moins) jusqu'au 60ème jour après l'opération.

Au niveau des ganglions des racines dorsales T9-T11, en arrière de la section, l'augmentation de l'expression d'OX-42 et d'ATF3 est transitoire. Au-delà de deux semaines après la lésion, leurs taux ne diffèrent plus de ceux des rats « sham ». Dans les ganglions T6-T8, en avant de la lésion, l'augmentation d'ATF3 se maintient pendant les 60 jours de l'étude tandis que celle d'OX-42 n'est présente que les 4 premiers jours après la section. Concernant GFAP, l'activation est transitoire. Elle n'est observée que jusque 9 et 15 jours après la section, respectivement au-dessus et en dessous de la section.

- *Les cytokines IL-6, IL-1β, IL-10, TNF-α*

Au niveau de la moelle épinière, les transcrits de ces 4 cytokines sont tous surexprimés, que ce soit en avant ou en arrière de la section, dès 2 jours après l'opération. Cette surexpression se

maintient mais à des niveaux différents d'une cytokine à l'autre jusqu'au 60ème jour après la lésion.
Au niveau des ganglions des racines dorsales, l'expression de l'ARNm de TNFα n'est pas augmentée chez les rats SCT. En revanche, celle des transcrits codant IL-1β et IL10 est induite, mais faiblement, à J2 et pas à J60. Par contre, on note une forte augmentation de l'expression d'IL6 au 2ème jour après la section. Cette induction d'IL-6 diminue progressivement mais les taux de son ARNm restent significativement plus élevés chez les rats SCT que chez les « sham » dans les ganglions rostraux (T6-T8) au moins jusque 21 jours après la section.

- *Les récepteurs gliaux P2X4, P2X7, TLR4*

Les ARNm codant les récepteurs purinergiques P2X4 et P2X7 et les récepteurs TLR4 de la microglie et des astrocytes montrent une forte augmentation à court et à long terme au niveau spinal, aussi bien dans les segments rostraux que dans les segments caudaux par rapport à la section. Au niveau des ganglions des racines dorsales, on n'observe pas de modifications des concentrations de ces transcrits, hormis une élévation dans le cas de l'ARNm P2X7 à J2 dans les ganglions rostraux (T6-T8).

III - DISCUSSION

Les rats présentent un bon état général après l'opération de section de moelle épinière. L'absence d'autotomie et la prise de poids au même rythme que chez les animaux contrôles laissent à penser que la section spinale au niveau thoracique est un modèle éthiquement recevable d'une douleur neuropathique d'origine centrale. Les examens pratiqués sur tout le territoire corporel montrent des modifications de la sensibilité mécanique seulement au niveau des pattes postérieures et d'une zone restreinte en avant et autour du site de l'intervention chirurgicale. L'hypersensibilité au niveau des pattes postérieures peut être assimilée au phénomène d'hyper-réfléxie qui est observé chez le patient médullo-lésé présentant une augmentation du réflexe H (Lotta et al., 1991; Calencie et al., 1993). Elle serait due à une hyperexcitabilité des motoneurones après lésion de la moelle épinière (Garrison et al., 2011). En revanche, aucune différence de sensibilité n'a été mise en évidence entre les animaux « sham » et les animaux médullo-lésés au niveau des pattes antérieures. Dans un territoire cutané limité (6 cm²) autour du site de l'opération, mais seulement du côté rostral, se développe une allodynie de longue durée

chez **100% des animaux médullo-lésés** par rapport aux animaux « sham ». Plusieurs auteurs ont rapporté une altération de l'activité électrophysiologique des neurones spinaux (Scheifer et al., 2002; Hoheisel et al., 2003) ainsi qu'une augmentation de l'activité électrique des nocicepteurs (Carlton et al., 2009) chez les rats médullo-lésés. Ces deux phénomènes de sensibilisation centrale et périphérique pourraient être impliqués dans l'induction et/ou le maintien de cette allodynie locale chez les rats SCT.

L'induction et le maintien de cette allodynie pourraient être d'origine gliale. De fait, chez les rats SCT, on observe une forte activation astrocytaire et microgliale qui se prolonge dans le temps en parallèle avec l'allodynie observée, comme cela a déjà été rapporté pour d'autres modèles de douleurs neuropathiques causées par une lésion de moelle épinière (Carlton et al., 2009). De plus, le blocage de l'activation gliale par l'injection intrathécale de propentofylline réduit l'allodynie au niveau des pattes postérieures chez les animaux médullo-lésés (probablement via un rétablissement de la transmission GABAergique ; Gwak et al., 2008). Bien que le rôle des récepteurs purinergiques P2X4 et P2X7 et du récepteur TLR4 dans l'allodynie après lésion spinale n'ait pas été formellement démontré, on peut supposer que ces récepteurs puissent être impliqués dans l'induction d'une douleur neuropathique qu'elle soit d'origine centrale ou périphérique (Wu et al., 2010; Inoue & Tsuda, 2012).

Les cytokines pro-inflammatoires dont l'expression est augmentée chez le rat SCT sont libérées par les cellules microgliales et astrocytaires activées et concourent aussi à l'établissement de douleurs neuropathiques centrales dans d'autres modèles de lésion de la moelle épinière (Gwak et al., 2011; Guptarak et al., 2013). Afin de déterminer précisément l'importance respective et le rôle de ces différents facteurs gliaux dans l'induction et le maintien de l'allodynie dans le territoire cutané autour de la lésion, il serait intéressant de bloquer leurs voies de signalisation en aval par des inhibiteurs spécifiques et d'en analyser les conséquences sur les réponses dans le test des filaments de von Frey.

Parmi tous les agents pharmacologiques testés, seuls la kétamine, le tapentadol, la morphine et, dans une moindre mesure, le baclofène, ont réduit l'allodynie au niveau de la lésion. Or, chez l'homme, la kétamine est efficace pour diminuer les douleurs neuropathiques centrales (Kim et al., 2013), de même que les composés opioïdes (Attal et al., 2010). Pour sa part, le baclofène est généralement utilisé pour atténuer les phénomènes de spasticité chez les patients médullo-lésés (Lewis & Mueller, 1993). La cohérence entre ces données et nos résultats atteste de la validité du modèle SCT sur le plan pharmacologique. De fait, l'inefficacité des autres composés, notamment ceux qui sont efficaces chez l'homme comme l'amitriptyline, peut être imputée à nos conditions

de traitements dans la mesure où ce n'est qu'à la suite d'une administration chronique qu'ils soulagent véritablement les patients neuropathiques de leurs hyperalgésie et allodynie.

IV- CONCLUSION

Le modèle de transection de la moelle épinière, bien que peu représentatif de la plupart des atteintes médullaires chez l'homme, semble constituer un modèle parfaitement valide pour l'étude de la douleur neuropathique centrale. Tout d'abord, ce modèle est parfaitement reproductible, induisant une allodynie **chez 100% des animaux opérés** dans toutes nos séries expérimentales. De plus, l'allodynie induite au niveau du territoire cutané juste en avant de la lésion est tout à fait **homologue de l'allodynie au niveau de la lésion chez l'homme**. Par ailleurs, les voies de signalisation cellulaires induites par la transection, et très probablement l'origine de l'allodynie, sont similaires à celles impliquées dans d'autres modèles de lésion de moelle. Enfin, plusieurs des agents pharmacologiques efficaces chez l'homme ont aussi prouvé leur efficacité chez le rat SCT, conférant à ce modèle un **caractère prédictif en ce qui concerne la pharmacologie**. En d'autres termes, le rat SCT pourrait constituer un « outil » à la fois reproductible et fiable pour la caractérisation du potentiel anti-allodynique de composés innovants dans le cadre du développement de nouvelles stratégies thérapeutiques des douleurs neuropathiques d'origine centrale.

Behavioral, physiopathological and pharmacological characterization of the rat model of central neuropathic pain caused by spinal cord *transection*

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Abstract

In humans, spinal cord lesions induce not only major motor and neurovegetative deficits but also severe neuropathic pain in about half of patients. Such chronic pain is mostly resistant to classical analgesics, and there is an eager need of novel, effective and well tolerated alleviating treatments. To this goal, novel animal models have to be developed. In this context, we investigated the mechanical allodynia consecutive to spinal cord injury in rats. Sprague Dawley male rats underwent complete thoracic cord (T8-T9) transection under deep isoflurane anaesthesia, and the resulting mechanical sensory alterations were assessed in all body territories using von Frey filaments from day 1 to day 60 post surgery. Acute treatments with various drugs with real or potential anti-allodynic properties were performed on day 30 post surgery, when mechanical sensory alterations had reached a plateau. Real-time quantitative RT-PCR determinations allowed measurements of various transcripts encoding markers of neuronal injury, microglia and astrocyte activation and pro- and anti-inflammatory cytokines in spinal tissues and dorsal root ganglia of spinal cord transected (SCT) rats.

Thoracic cord transection induced a marked hyper-reflexia at hindpaws and a strong mechanical allodynia in a limited territory at the level of the surgery, with a pressure threshold to trigger nocifensive reaction to locally applied von Frey filaments 100-fold lower in SCT- versus sham-operated rats. A marked up-regulation of mRNAs encoding ATF3 (neuronal injury) and glial activation markers (OX-42, GFAP) was observed in spinal cord tissues and/or dorsal root ganglia at T6-T11 levels from day 2 up to day 60 post surgery. Transcripts encoding IL-1β, IL6 and TNF-α were up-regulated only transiently. Acute treatment with ketamine (50 mg/kg i.p.), morphine (3-10 mg/kg s.c.) and tapentadol (10-20 mg/kg i.p.) significantly increased pressure threshold to trigger nocifensive reaction in the von Frey filaments test, whereas amitriptyline, pregabalin, gabapentin and clonazepam were ineffective. Because all SCT rats developed long lasting, reproducible and stable allodynia, which could be alleviated by drugs effective in humans, transection of thoracic cord at T8-T9 might be a useful model for testing innovative treatments aimed at reducing spinal cord lesion-induced central neuropathic pain.

Key words: neuropathic pain, thoracic cord transection, von Frey filaments, mechanical allodynia, neuroinflammatory markers, alleviating drugs

Introduction

Spinal cord injury (SCI) is a debilitating state which occurs at an annual rate of 20-40 individuals over one million. In addition to severe motor dysfunctions, loss of bladder control and impairment of sexual function, more than 80% of the patients suffer from pain, part of them (40%) from neuropathic pain (Donovan et al., 1982; Finnerup et al., 2001; Bryce et al., 2006, 2012). Indeed, pain can be so severe that some patients would be ready to privilege pain relief at the expense of further deficits in bladder control or sexual function. SCI-induced central neuropathic pain can be localized above-, at- or below- the level of injury and is mostly characterized by allodynia refractory to conventional treatments.

Several animal models of SCI-induced neuropathic pain have been developed (through spinal cord transection, compression…etc) (Nakae et al., 2011), each of them displaying different characteristics in terms of localization, duration, type of pain and even response to drugs. Although some studies did provide relevant data regarding treatment efficacy and molecular mechanisms (Yezierski, 2000; Baastrup et al., 2010, 2011), they focused mostly on pain below lesion produced by contusion or clip compression of the spinal cord. Yet, despite the fact that these SCI models reproduce adequately some types of spinal cord injuries seen in humans, they have major limitations because of unavoidable large interindividual differences in the extent and severity of evoked lesions (Basso et al., 1996). Furthermore, lesion-induced neuroinflammatory processes appeared highly variable from one SCI rat to another (Crown et al., 2006), which makes studies of physiopathological mechanisms underlying SCI-induced neuropathic pain in these models especially difficult and poorly reliable.

In the present study, we investigated whether the rat model consisting of the complete transection of the spinal cord at the thoracic level could provide more reproducible data regarding SCI-induced central neuropathic pain, underlying mechanisms and responses to drugs with potential alleviating properties. We first characterized mechanical allodynia-related behavior in those animals, by assessing their responses in the von Frey filaments test. We then investigated the effects of acute treatments with various drugs (opioids, antidepressants, anticonvulsants and others) on mechanical allodynia in spinal cord transected (SCT) rats. Finally, we analyzed by real time semi-quantitative RT-PCR, at different times after the thoracic cord transection, the expression of mRNAs encoding proteins implicated in neuroinflammation and neuroplasticity, with particular focus on markers of microglia and astrocyte activation, pro- and anti-inflammatory cytokines (IL-1β, IL6, TNFα, IL10), trophic factors such as Brain Derived Neurotrophic Factor (BDNF) and nociceptive signaling pathways in dorsal root ganglia (DRG) and spinal cord tissues, for comparison with previous studies aimed at unveiling physiopathological mechanisms associated with neuropathic pain in other SCI models.

Materials and methods

Animals

Male Sprague–Dawley rats, weighing 225–250 g on arrival, were purchased from Janvier Breeding Center (53940 Le Genest Saint Isle, France). They were housed under standard controlled environmental conditions (22 ± 1°C, 60% relative humidity, 12:12 h light–dark cycle, lights on at 7 :00 am), with food and water available *ad libitum*. Rats were allowed to habituate to the housing facilities without any handling for at least 1 week before being used. In all cases, experiments were performed in conformity with the Ethical Guidelines of the Committee for Research and Ethical Issues of the International Association for the Study of Pain (Zimmermann, 1983) and strictly followed the Institutional Guidelines that are in compliance with French and international laws and policies (Council directive 87-848, October 19, 1987, Ministère de l'Agriculture et de la Forêt, Service vétérinaire de la santé et de la protection animale, permissions nb A752128 to S.M .nb 006228 to S.B., nb 00482 to V.K., nb 75 116 to M.H.).

Spinal cord transection

Animals underwent surgery under deep isoflurane anaesthesia (3%). Paravertebral muscles were cut and the T5 vertebra was opened. Local anaesthesia was made by cooling the spinal cord with cryoflurane (Promedica, France) a few seconds before the lesion. Complete transverse section with ophthalmic scissors at the T8-T9 spinal cord segments level was performed following the procedure described by Antri et al. (2005), then sterile absorbable haemostatic gel foam (Surgicel; Ethicon, Somerville, NJ, USA) was inserted into the lesion. Sham-operated animals underwent laminectomy only. At the last step of surgery, muscles were sutured and the skin was closed up by skin clips. Both SCT and sham-operated rats then received antibiotic treatments to prevent staphylococcic infection (oxacillin, Bristopen, Bristol Myers Squibb S.P.A., Italy, 0.3 mg/100 g s.c. once a day during 7 days) and urinary infection (gentamicin; Panpharma, France, 0.2 mg/100 g s.c., immediately after the surgery).

For recovery, SCT and sham rats were housed at 2 per cage. The bladder of SCT rats was emptied manually each day until reappearance of the voiding reflex (usually on the 10^{th} day post surgery) (see Results).

Tests with von Frey filaments

Assessment of at-level mechanical allodynia

For assessment of SCT-induced neuropathic-like pain in the cutaneous territory around surgery scar, rats were placed individually into a plastic cage (42x24x15 cm) and allowed to adapt to this environment for 1 hour before any stimulation. Tactile allodynia was then determined with a graded series of von Frey

filaments (Bioseb, 92370 Chaville, France) producing a bending force ranging between 0.008 g and 100 g. The stimuli were applied 3 times (3 seconds apart) for each filament, always beginning with the filament producing the lowest force, within a cutaneous territory of about 6 cm² around the lesion. Positive nociceptive behaviors consisted of either a shake, an attack (filament biting), or an escape reaction (Baastrup et al., 2010). The minimal force causing at least one of these responses allowed the determination of the mechanical pressure threshold. The 100 g filament, chosen as cut-off to prevent tissue injury, induced no nociceptive behavior in the majority (>90%) of naïve rats. To avoid nonspecific responses, only these "non-reactive" rats were selected for surgery and included in the study.

Assessment of mechanical sensitivity in body territories outside the allodynic area

SCT and sham-operated rats were also subjected to mechanical stimulation with von Frey filaments to assess evoked responses at the level of forepaws, hindpaws and body territories outside the 6 cm² area around the lesion. For these tests, they were placed on a wire grid platform (5 x 5 mm mesh) under small plastic (35 x 20 x 15 cm) cages for 2 hours, and mechanical sensitivity was determined with a graded series of 9 von Frey filaments (bending force of 4, 6, 8, 10, 12, 15, 26, 60 and 100 g). At paw level, stimuli were applied onto the lateral plantar surface of the right forepaw or hindpaw 3 times (3 seconds apart) for each filament, always beginning with the filament producing the lowest force. The minimal force filament for which animals presented either a brisk paw withdrawal and/or an escape attempt allowed determination of the mechanical pressure threshold (Latrémolière et al., 2008). Usually, the pressure threshold value to trigger a (non nocifensive) response in naïve healthy rats was around 60 g. Because SCT rats presented large time-dependent changes in mechanical sensitivity (see Fig. 1), higher pressures were also tested, with cut-off fixed at 100 g to avoid any tissue injury.

Pharmacological treatments

Gabapentin and pregabalin were purchased from Sequoia (Pangbourne, UK). Baclofen, amitriptyline, ketamine and 8-OH-DPAT [(±)-8 hydroxy-2-dipropylamino-tetralin] were from Sigma-Aldrich (Saint Quentin Fallavier, France). Other compounds were cyclotraxin B (BIO S&T, Montreal, Canada), tapentadol (Grünenthal, Aachen, Germany), morphine (Pharmacie Centrale des Hôpitaux de Paris, France), naratriptan and ondansetron (Glaxo Wellcome, Harlow, UK), and clonazepam (Roche, Basel, Switzerland).

Routes of administration and doses (as free bases; see Table 1) were chosen according to previous data in the literature. All drugs were dissolved in saline (0.9% NaCl) except baclofen which was dissolved in DMSO:0.9% NaCl (50:50) and clonazepam which was suspended in ethanol:water (50:50). Drugs or their vehicles were injected acutely 30 days after transection, when mechanical allodynia had fully developed in the 6 cm² area around the surgery scar (see Results). For intrathecal injections (of ondansetron), rats

were briefly anaesthetized with isoflurane (3% in air), and the needle (26 G) was inserted into the lumbar space between the L5 and L6 vertebrae (Mestre et al., 1994) for administration of the appropriate dose in 20 µL of saline. Von Frey filaments test was then applied at various times after acute treatment to determine the time course of drug-induced changes in pressure threshold to trigger nocifensive response (biting of the filament, see Results), until the drug effect completely disappeared.

Real time semi-quantitative RT-PCR measurements

SCT- and sham-operated or control rats were sacrificed at various times, from 2 to 60 days after surgery. Dorsal root ganglia (DRG), thoracic cord segments below (T9-T11) and above (T6-T8) the lesion, along with cervical and lumbar enlargements, were rapidly dissected out at 0-4 °C, and immediately frozen in liquid nitrogen to be stored at -80 °C. Total RNA was extracted using the NucleoSpin RNA II extraction kit (Macherey-Nagel, 67722 Hoerdt, France) and quantified using NanoDrop. First-stranded cDNA synthesis (from 660 ng total RNA per 20 µl reaction mixture) was carried out using High Capacity cDNA reverse transcription kit (Applied Biosystems, Courtaboeuf, France). PCR amplification, in triplicate for each sample, was performed using ABI Prism 7300 (Applied Biosystems, Courtaboeuf, France), TaqMan® Universal PCR Master Mix No AmpErase® UNG (Applied Biosystems) and Assays-on-Demand Gene Expression probes (Applied Biosystems) for targets gene: *ATF3* (assay ID Rn00563784_m1), *GFAP* (Rn01460868_m1), *OX42* (Rn00709342_m1), *IL-1β* (Rn00580432_m1), *IL6* (Rn00561420_m1), *TNF-α* (Rn00562055_m1), *IL-10* (Rn00563409_m1) *BDNF* (Rn02531967_s1), *TLR4* (Rn00569848_m1), *P2X4* (Rn00580949_m1), *P2X7* (Rn00570451_m1). Semi-quantitative determinations were made with reference to the reporter gene encoding glyceraldehyde 3-phosphate dehydrogenase (*GaPDH*; Rn99999916_s1). The polymerase activation step at 95°C for 15 min was followed by 40 cycles of 15 s at 95°C and 60 s at 60°C. The validity of the results was checked by running appropriate negative controls (replacement of cDNA by water for PCR amplification; omission of reverse transcriptase for cDNA synthesis). Specific mRNA levels were calculated after normalizing from *GaPDH* mRNA in each sample. Data are presented as relative mRNA units compared to control values (see Latrémolière et al., 2008).

Statistical analyses

All values are expressed as means ± S.E.M. For behavioral tests, the data were analyzed by one-way ANOVA for repeated measures (effect of a drug over time) followed by a Dunnett's test. For RT-PCR data, the $2^{-\Delta\Delta Ct}$ method (Schmittgen and Livak, 2008) was used for the analysis of the relative changes in specific mRNA levels and for the graphic representations (RQ Study Software 1.2 version; Applied Biosystems). For analysis of the time course expression of the target genes, a two-way ANOVA was

performed followed by a Bonferonni test for comparison of SCT rats versus their respective control at each time. The critical level of significance was set at $P < 0.05$.

Results

Physiological state of spinal cord transected rats

After full recovery from anaesthesia, SCT rats first showed hindlimb paralysis and flabbiness. Although they moved in their cage without major difficulty and could access food and water as readily as before the surgery, SCT rats stopped gaining weight for the first week after surgery (-6.3 ± 3.8 g, mean ± S.E.M., n = 8) , in contrast to sham-operated animals (+43 ± 2 g, mean ± S.E.M., n = 8); but, afterwards, weight gain was parallel in both SCT- and sham-operated rats (+ 175.4 ± 28.3 g and + 171.2 ± 12.5 g from day 7 to day 30 post-surgery, respectively, means ± S.E.M., n = 8 in each group).

Most striking symptoms were urinary retention and/or hematuria. Hematuria disappeared after 3 or 4 days without any specific treatment. To deal with urinary retention, we had to provoke the miction reflex by rubbing the bladder once a day during 8-9 days on average. Then, the reflex recovered completely. It also happened that some SCT rats had an accelerated gut transit with diarrhea for the first 3 days post surgery. Then, such gut disorders were only exceptionally observed. On the other hand, SCT rats had their fur a little bit more tousled than sham animals, but it stayed very clean above and below the lesion site, probably thanks to their cage mate.

Immediately after the surgery and during usually 9 days, SCT rats showed paraplegia, first characterized by a total absence of reaction when hindlimbs were mechanically stimulated with von Frey filaments exerting pressure up to the cut-off value (100 g) (Fig. 1). This was followed by a hypo-reflexia which progressively vanished up to normal-like response to mechanical stimulation which was usually recovered two weeks post surgery. Later on, SCT rats developed a hyper-reflexia with a pressure threshold value to trigger brisk hindpaw withdrawal strikingly lower (-80%) than that determined in sham rats up to two months post-surgery (Fig. 1). All along the observation period, SCT rats had paralyzed hindlimbs with spasticity, rigidity and tonicity. They also had frequent spontaneous movements of the tail and hindlimbs (shaking), and developed uncoordinated flexion and extension movements. We never observed autotomy.

Development and localization of mechanical allodynia

Among all the body areas tested, only the lesion site on the back and the hindlimbs (see above) showed altered behavioral responses in the von Frey filaments test in SCT- compared to sham-operated rats.

Within a few days after SCT, supersensitivity to mechanical stimulation appeared at the level of surgery scar. From day 2 to day 9, such supersensitivity was mostly lateral to the lesion site within small areas on both sides (Fig. 2). Then, the supersensitive territory extended medially and laterally to cover an approximately 6 cm^2 area surrounding the lesion site, just above the thoracic cord transection. In

contrast, no supersensitivity was detected below the transection, and, indeed, SCT rats did not react even to a 100 g pressure exerted by a von Frey filament applied within the cutaneous territory below the transection.

Further assessment of supersensitivity to application of von Frey filaments within the 6 cm^2 area around surgery scar led to identify three different aversive reactions: shaking, biting and escape (Fig. 3), in agreement with previous observations in SCI rats (Baastrup et al., 2010). Determinations of pressure thresholds to trigger each of these behaviors showed parallel time-course decreases, down to very low values that were reached 10-14 days after surgery and remained unchanged for the 7-weeks-observation period (Fig. 3).

Pharmacological studies

Effect of opioïdergic drugs (morphine and tapentadol) on at-level mechanical allodynia

As treatments with opioids were shown to reduce pain in humans with spinal cord lesions (Fenollosa et al., 1993; Norrbrink and Lundeberg, 2009), we investigated whether morphine (1, 3 and 10 mg/kg s.c.) was effective to reduce at-level allodynia in SCT-rats. Acute treatment was performed 30 days after the surgery, when pressure threshold to elicit biting behavior in response to von Frey filament application had reached its minimum value (Fig. 3). As illustrated in Figure 4A, morphine exerted a dose-dependent effect: it was inactive at 1 mg/kg s.c., but increased pressure threshold value at higher doses, with complete suppression of allodynia-like response 30 and 60 min after administration of the highest dose tested (10 mg/kg s.c.). Confirmation of the anti-allodynic efficacy of opiate receptor activation was made with tapentadol, a mixed mu opioid receptor agonist and noradrenaline reuptake inhibitor with potent antalgic properties (Tzschentke *et al.*, 2007), which also reversed SCT-induced mechanical allodynia in a dose-dependent manner. As shown in Figure 4B, tapentadol at 10 mg/kg i.p. slightly increased the pressure threshold value, but the dose of 20 mg/kg i.p. completely suppressed allodynia-like response 30 and 60 min after its administration to SCT rats.

Effect of ketamine on at-level mechanical allodynia

Ketamine is well known to reduce pain in humans suffering from spinal cord injury (Kim et al., 2013), and its pain alleviating efficacy has also been reported in SCI models, such as the one obtained by spinal cord contusion (Bennett et al., 2000). In our SCT model, acute administration of ketamine (50 mg/kg i.p.) induced a significant increase in pressure threshold value to trigger nocifensive response to von Frey filament application within the allodynic cutaneous area. At its maximum, 30 min after treatment, pressure threshold reached 77.3 ± 17.6 g (from 0.96 g ± 0.39 g before treatment, means ± S.E.M. of 6 determinations), which was not significantly different from the cut-off value corresponding to the non-

allodynic state (in naïve rats, before surgery). However, this effect vanished rapidly because mechanical allodynia was completely restored 90 min after ketamine administration (Fig. 4C).

Effects of baclofen on at-level mechanical allodynia

Because baclofen, a GABA B receptor agonist, is often prescribed to reduce SCI-induced spasticity in humans, and is endowed with anti-neuropathic pain properties (Gwak et al., 2006), we investigated whether this drug could reduce SCT-induced mechanical allodynia in rats. Indeed, baclofen induced a limited and transient increase ($P < 0.05$) in pressure threshold value, from 0.6 ± 0.4 g before treatment to 5.0 ± 2.1 g 30 min after i.p. administration of this drug at the dose of 10 mg/kg (Fig. 4D).

Effects of anticonvulsant drugs on at-level mechanical allodynia

The calcium channel blockers pregabalin and gabapentin and the benzodiazepine clonazepam are anticonvulsants endowed with anti-neuropathic pain properties both in humans (Fenollosa et al., 1993; Attal et al., 2010) and in rodent models (Wallin et al., 2002), and we therefore tested whether these drugs also exerted anti-allodynic effects in SCT rats. However, acute treatments with either pregabalin (30 mg/kg i.p.) gabapentin (30 mg/kg i.p.) or clonazepam (0.25 mg/kg i.p.), at doses devoid of any inhibitory effect on locomotor activity, had no significant effect on pressure threshold to trigger nocifensive response in SCT rats (Table 1). Some increase was noted with higher doses of clonazepam (2 mg/kg i.p.) and gabapentin (100 and 300 mg/kg i.p.), but animals presented profound ataxia after such treatments (not shown).

Effects of other drugs on at-level mechanical allodynia

As detailed in Table 1, the antidepressant amitriptyline, alone or combined with gabapentin, the anti-migraine drug naratriptan, the 5-$HT_{1A/7}$ receptor agonist 8-OH-DPAT, the 5-HT_3 receptor antagonist ondansetron, the BDNF-Trk B receptor blocker cyclotraxin B, at effective doses to reduce pain in validated neuropathic models in rodents (Suzuki et al., 2004; Kayser et al., 2002, 2010; 2011; Constandil et al., 2012 ; Vanelderen et al. 2013), exerted no anti-allodynic effects up to 3 hours after acute administration in SCT rats.

Neuroinflammatory and neuroplasticity markers in DRG and spinal cord tissues of SCT rats

Dorsal root ganglia

The transcript encoding the neuronal injury marker ATF3 was strongly up-regulated in DRG at T9-T11 below-level as well as T6-T8 above-level of injury at days 2 – 9/15 after thoracic cord transection (Fig. 5A). Then, significant increases persisted up to the last observation day, two months after surgery, but to a lower extent, only in T6-T8 DRG (Fig. 5A). Transcripts encoding glial cell activation markers, OX-42

(microglia) and GFAP (astrocytes), were also markedly up regulated in DRG at spinal segments below (T9-T11) and above (T6-T8) the transection. However, this effect was transient, especially above level (T6-T8) where significant increases in OX-42 and GFAP transcripts were noted only on day 2 and up to day 9 post-surgery, respectively. In below level (T9-T11) DRG, up regulation of these transcripts lasted a few days more, but three weeks post-surgery, both OX-42 and GFAP transcripts no longer differed in thoracic DRG of SCT- versus sham-rats (Figs 5B,5C).

Transcripts encoding pro-inflammatory cytokines were also markedly, but differentially, affected in thoracic DRG of SCT rats. Thus, mRNA encoding the pro-inflammatory cytokine IL-6 showed a dramatic up-regulation (x 65.6) in T9-T11 DRG at day 2 post surgery (Fig. 6A). Its levels then decreased rapidly, but remained significantly higher than in sham rats up to day 9 post surgery (x 4.4). Interestingly, up-regulation of IL-6 mRNA was even larger at day 2 (x 149.0) and remained significant for a longer period (up to day 50 post surgery: x 1.6) in above level T6-T8 DRG (Fig. 6A). An up regulation of IL-1β mRNA was also noted in thoracic DRG at day 2 post-surgery (but not at day 60) in SCT rats (Fig. 6B), but this change was of much lower amplitude (x 6.2 in T9-11 DRG, x 4.6 in T6-T8 DRG, as compared to respective levels in sham rats) than that of IL-6 mRNA. In contrast with the transcripts encoding the latter two pro-inflammatory cytokines, TNF-α mRNA was not up-regulated in thoracic DRG of SCT rats, neither at day 2 nor at day 60 post-surgery (Fig. 6C). Finally, the levels of mRNA encoding the anti-inflammatory cytokine IL-10 were found to be slightly increased (x 3.1), but only in DRG below the section (T9-T11) on day 2 post surgery (Fig. 6D).

Complementary RT-PCR determinations showed that BDNF mRNA levels were significantly increased in below-level T9-T11 DRG at both days 2 (x 5.6) and 55 (x 1.6) post-surgery, but only at day 2 (x 4.3) in above-level T6-T8 DRG (not shown). On the other hand, mRNAs encoding P2X4, P2X7 and TLR4, which are all expressed by activated glial cells (Inoue, 2002, 2006; Fellner et al., 2013), showed no modification of their expression levels in DRG whatever the time after SCT (Figs. 7A, 7B, 7C).

Spinal cord

The expression of the same markers was studied in central (spinal) tissues, both at the level of the section (thoracic spinal cord below and above the lesion), and in remote segments corresponding to the cervical and lumbar enlargements.

As in corresponding DRG, ATF3 mRNA was strongly up-regulated in spinal segments just above and below transection (Fig. 5A). Thus, on day 2 post-surgery, ATF3 mRNA levels were 17.0- and 22.0-fold higher in thoracic spinal cord below and above the section, respectively, than in corresponding tissues from sham-operated rats. This up-regulation was long lasting as it persisted, but to a lower extent, up to the last observation day (x 7.0 and 6.7 on day 60 post-surgery) (Fig. 5A). As illustrated in Fig. 5A, a long lasting up regulation of ATF3 mRNA was also detected in both the cervical and lumbar enlargements of

the spinal cord in SCT rats. However, this change was of much lower amplitude than in thoracic segments. OX-42 mRNA levels were also markedly increased in thoracic segments of the spinal cord just below and above the section on day 2 post-surgery (x 6.5 and 4.8, respectively), and remained significantly elevated until day 60 (x 2.8 and 2.5, respectively) (Fig. 5B). A long lasting up-regulation of OX-42 mRNA was also noted in both the cervical and lumbar enlargements of the spinal cord. However, it was of lower amplitude than in thoracic segments (Fig. 5B). The time course of SCT-induced changes in GFAP mRNA levels differed from those of the former two transcripts, as up-regulation was delayed and reached statistical significance only at post-surgery day 21 below the section (x 3.2), and at day 4 above the section (x 1.8) (Fig. 5C). Furthermore, these changes persisted to similar extents up to the last observation day (day 60 post surgery). In cervical and lumbar enlargements, only slight, generally non significant, increases in GFAP mRNA levels were observed in SCT rats, but they were also of long duration (Fig. 5C).

Concerning cytokines, a massive increase in IL6 mRNA levels was observed 2 days after the section in thoracic segments below (x 76.8 as compared to sham-operated rats) and above (x 66.4) the section (Fig. 6A). A modest up-regulation was still observed on day 15 but not on day 60 post surgery. In contrast, no significant changes in IL6 mRNA levels were detected in both the cervical and lumbar segments of the spinal cord at any time after SCT as compared to transcript levels measured in the same tissues of sham-operated rats (not shown).

The levels of IL-1ß mRNA were also markedly increased 2 days after surgery in thoracic segments below (x 172.2 as compared to sham-operated rats) and above (x 102.6) transection (Fig. 6B). A modest but significant increase was also detected in below level segments on day 60 post-surgery, but to a much lower extent. Similar but less pronounced changes in TNF-α mRNA levels were noted with a significant up-regulation in thoracic segments on day 2 post surgery (x 3.0 below and x 1.9 above the section, respectively) (Fig. 6C). On day 60, a significant increase in TNF-α mRNA levels was still detected principally in thoracic segments above transection (x 1.9) (Fig. 6C). Finally, tissue concentrations of mRNA encoding the anti-inflammatory cytokine IL-10 were also markedly increased on day 2 after transection in both below-level (x 36.3) and above-level (x 38.7) thoracic segments, and an up-regulation of much lower amplitude was still detected on day 60 post surgery (Fig. 6D).

In sharp contrast with that observed in thoracic DRG, BDNF mRNA levels were reduced in spinal cord tissues of SCT rats, both on days 2 (-49% as compared to sham-operated rats) and 60 (-38%) post-surgery in thoracic segments below the section and on day 60 (-23%) in thoracic segments above the section (not shown). On the other hand, mRNAs encoding P2X4, P2X7 and TLR4 were up-regulated in thoracic segments below (x 3.4, x 1.8 and x 3.8, respectively) and above (x 2.6, x 1.5 and x 3.6, respectively) transection on day 2 post-surgery. This up-regulation was even more pronounced on post-

surgery day 60 (x 3.6, x 2.9 and x 4.5 below the section, x 3.9, x 2.9 and x 5.8 above the section, respectively) (Figs. 7A, 7B, 7C).

Discussion

Several rodent models of spinal cord lesions (contusion, photochemically induced, ischemia, hemisection) have been developed and validated especially for the study of spinal mechanisms of locomotion (Boulenguez and Vinay, 2009; Rossignol and Frigon, 2011). Occasionally, these models have also been used to investigate physiopathological mechanisms underlying the resulting central neuropathic pain. However, most of these models generate heterogeneous data because of unavoidable inter-individual differences in the severity of lesion from one rat to another, which lead to a high degree of variability in neuroinflammation patterns and downstream hyperalgesia and allodynia (Basso et al., 1996). Indeed, some authors had to distinguish sub-groups with different levels of neuropathic pain in rats subjected to the very same procedure to generate neuropathic pain (Crown et al., 2006). With the objective of raising more reliable homogeneous data, we chose to better characterize neuropathic pain generated by spinal cord transection, because this model implies a highly reproducible lesion in terms of severity. Furthermore, to date, only few studies have been specifically devoted to the characterization of central neuropathic pain in rats.

Clinical state of spinal cord transected rats

Despite complete transection of the spinal cord, rats showed a relatively good physiological state. The lack of micturition reflex and the hematuria, which are commonly encountered in paraplegic patients (Singh et al., 2011), usually resolved within 9 days post-surgery. Otherwise, their fur was clean, and, as long as they shared their cage with a congener, autotomia never occurred. Although rats lose weight for the first week after surgery, probably because of hindlimb muscles atrophy, they subsequently gained weight at the same rate as sham animals, as expected from animals in good health (Ramsey et al., 2010).

Effect of spinal cord transection on hindlimb sensitivity

Just after the lesion, hindlimbs no longer responded by a reflex motor reaction to cutaneous mechanical stimulation at high intensity (with the 100 g von Frey filament). Motor reaction then reappeared progressively up to a level corresponding to that found in sham-operated animals around the second week post-surgery. A marked hyper-reflexivity subsequently developed progressively, along with spasticity, which reached their maximum approximately 7 weeks post-surgery and were still fully present on the last day (60) of our study. Marked alterations of motor reflexes also occur in humans with complete spinal cord transection, as evidenced by the exacerbated response in the H reflex of hindlimb muscles (Lotta et al., 1991; Calancie et al., 1993). Such facilitated reflex responses may be due to α-motoneurons hyperexcitability (Garrison et al., 2011). Indeed, spinal cord transection causes an up-regulation of constitutively active 5-HT_{2C} receptors expressed by motoneurons, and the reinforcement of their membrane depolarizing influence has been demonstrated to contribute to motoneuron

hyperexcitability in lesioned rats (Murray et al., 2010). On the other hand, spasticity could be accounted for by a down regulation of the potassium-chloride transporter KCC2 within the lumbar spinal cord below transection (Boulenguez et al., 2010). Although spasticity can be painful in humans, and below-level pain exists in humans with extensive spinal cord injury (Werhagen et al., 2004; Bryce et al., 2012), hyper-reflexivity and spasticity at hindlimb level could not be related to pain behavior in SCT rats, because completeness of the lesion necessarily prevented the nociceptive message to reach the sensory cortex where it can generate pain sensation.

At-level allodynia

Whereas no reaction of the animal to the application of von Frey filaments within the trunk below the lesion could be elicited in SCT rats, at-level allodynia-like reactions appeared relatively rapidly and reached a maximum 30 days after surgery. Whereas trunk shakes can be associated with spinal reflexes, but are not directly correlated with pain, biting is considered as a brainstem response and escape as a cortical response (Baastrup et al., 2010). Accordingly, the latter two responses were very probably associated with pain in SCT rats.

Since sham-operated rats did not develop such behaviors, we can exclude that they might have corresponded to musculoskeletal pain. Instead, at-level mechanical allodynia pain was very probably caused by spinal cord injury itself, as expected of neuropathic pain of central (spinal) origin (Bryce et al., 2012).

Interestingly, 100% of SCT rats developed at-level allodynia, contrary to humans with spinal cord lesion and rats with spinal cord contusion as only a fraction of lesioned subjects suffer from such pain symptoms. Indeed, the prevalence for the rat/human to develop at-level pain depends on the extent of the lesion (Hulsebosch et al., 2009). In SCT rats with complete spinal cord transection, basal electrophysiological activity of neurons rostral to the lesion is pathologically altered, with marked changes in impulse flow pattern. Actually, these modifications are correlated with allodynia, leading lesioned animals to perform excessive grooming in dermatomes close to the lesion (Scheifer et al., 2002). In contusion models of spinal cord injury, at-level pain was shown to involve hyper-excitability of nociceptors (Carlton et al., 2009). This hyper-excitability is unlikely to be sustained by functional modifications of ion channels. Indeed, blockers of voltage-dependent sodium channels such as lidocaïne cannot reduce at-level pain in spinal cord injured patients (Attal et al., 2010; Dworkin et al., 2010).

Pharmacological sensitivity of at-level allodynia in SCT rats

Only a few drugs among those tested were found to efficiently reduce at-level allodynia when injected acutely in SCT rats (see Table 1). The efficacy of morphine and tapentadol was probably underlain by the capacity of mu opioid receptor activation to inhibit the activity of wide dynamic range neurons in the

dorsal horn of the spinal cord (Wang et al., 2005). Interestingly, tapentadol had a somewhat more prolonged effect than morphine, may be because of its additional capacity to inhibit noradrenaline reuptake as this monoamine has been shown to be implicated in descending inhibitory control of neuropathic pain (Millan, 2002).

Ketamine also reversed at-level allodynia in SCT rats, in consistence with human data that demonstrated that this NMDA receptor antagonist is especially efficient to reduce allodynia in spinal cord injured patients (Kim et al., 2013). This marked effect of ketamine, that may be sustained by a temporary inhibition of astrocyte activation, further supports the key role played by glutamate receptors, particularly NMDA receptors, in physiopathological mechanisms underlying neuropathic pain (Niesters and Dahan, 2012).

Finally, the last drug of the series tested which was found to exert some (but modest) anti-allodynic effects in SCT rats was the GABA B receptor agonist, baclofen, commonly used to suppress spasticity in spinal cord injured patients. Spinal cord injury is known to be associated with a decreased tone of inhibitory GABAergic neurotransmission (Zhang et al., 1994; Yezierski, 2000), and it can be proposed that baclofen transiently compensated for this deficit, thereby reducing allodynia in SCT rats. In contrast, clonazepam, which is used to alleviate SCI patients from neuropathic pain (Fenollosa et al., 1993), was inefficient suggesting that GABA A receptor activation was ineffective to inhibit at-level allodynia in SCT rats (Table 1).

Serotonin is known to play a major role in pain control via the activation of several receptor types (Kayser et al., 2010). Thus, F13640, a potent and selective 5-HT_{1A} receptor agonist, appeared to be especially effective to suppress allodynia in spinal cord lesioned rats (Colpaert et al., 2004). In our hands, the prototypical 5-HT_{1A} receptor agonist, 8-OH-DPAT, did not reduce allodynia in SCT rats. Yet this molecule is also an agonist at 5-HT_7 receptors, whose activation can result in effects opposite to that expected from 5-HT_{1A} receptor activation (Amaya-Castellanos et al., 2011). Further studies with selective 5-HT_{1A} and 5-HT_7 receptor ligands have therefore to be performed in order to reach a clear-cut conclusion regarding the potential 5-HT modulations of at-level allodynia through these receptors.

Because allodynia-like sensory dysfunctions are associated with migraine (Aguggia et al., 2013), we investigated whether the anti-migraine drug, naratriptan, with potent 5-$HT_{1B/1D}$ receptor agonist properties could alleviate at-level allodynia in SCT rats. Indeed, no effect was observed, possibly because triptans were found to selectively reduce neuropathic pain at cephalic level but not in extra-cephalic territories (Kayser et al., 2002). Finally, the last 5-HT receptor that we selected for our pharmacological investigations was the 5-HT_3 type whose implication in modulatory controls of neuropathic pain has been firmly established (McCleane et al., 2003). Indeed, spinal 5-HT_3 receptor blockade by i.t. administered ondansetron was reported to attenuate, at least partially, neuropathic pain in a model of spinal contusion (Chen et al., 2009). In contrast, i.t. injection of ondansetron was inactive in SCT rats,

probably because the complete transection of the spinal cord suppresses the bulbo-spinal connections that mediate the alleviating effect of 5-HT_3 receptor ligands.

Under our acute treatment conditions, neither the antidepressant amitriptyline nor the anticonvulsants gabapentin and pregabalin, which are commonly used to reduce neuropathic pain in spinal cord injured patients (Attal et al., 2011), exerted any significant anti-allodynic effect in SCT rats (Table 1). Indeed, numerous studies showed that these drugs are effective only under chronic treatment conditions (Tzellos et al., 2008; Vanelderen et al., 2013), and further experiments consisting of repeated administrations of antidepressants and anticonvulsants have to be performed before concluding about their effectiveness or ineffectiveness in the SCT rat model. At present, the question remains open because pregabalin has been shown to act on the affective, cerebral cortex-mediated, component of pain in spinal cord-lesioned rats (Baastrup et al., 2011), whereas the tests used in our studies can only assess spinal- and brainstem- mediated responses to pain.

Finally, because BDNF and its receptor TrkB play key roles in physiopathological mechanisms underlying neuropathic pain (Merighi et al., 2008; Trang et al., 2011), we investigated whether acute TrkB blockade by cyclotraxin B could affect allodynia in SCT rats. Indeed, Constandil et al. (2012) reported that this drug can prevent and reverse neuropathic pain caused by peripheral nerve ligation in rats. In contrast, we found that cyclotraxin B was unable to reduce allodynia in SCT rats. However, it has to be emphasized that cyclotraxin B was administered at a time (30 days post-surgery) when BDNF mRNA was not longer up regulated (see Results), with, in turn, TrkB receptor no longer hyper-activated. In future studies, it will be of particular interest to assess the effect of cyclotraxin B as soon as 2 days after the surgery, at a time when BDNF mRNA was markedly up-regulated in thoracic DRG of SCT rats.

Neuroinflammation and glial activation in SCT rats

The transcription factor ATF3 is enhanced when neurons are injured or stressed, and implicated in regeneration and plasticity (Latrémolière et al., 2008). Its role in the maintenance of central neuropathic pain is the matter of controversy, as it is no longer expressed when pain is still present after spinal cord injury (Carlton et al., 2009). However, its implication in the induction of central neuropathic pain is clearly established, since ATF3 participates in the activation of the microglial marker OX-42 and the astrocyte marker GFAP (Hai and Hartman, 2001; Block et al., 2007), two factors whose expression is closely associated with neural lesion-evoked neuropathic pain (Gwak et al., 2008, 2012; Latrémolière et al., 2008; Carlton et al., 2009; Kim et al., 2013; Tsuda et al., 2013). Because ATF3 activation is triggered by cellular damages, and this transcription factor is able to repress the activity of its own promoter (Hai and Hartman, 2001), the long lasting up-regulation of ATF3 transcript that occurred after SCT very probably reflected an ongoing neuronal damage. Convergent data in the literature showed that microglia activation is mediated, among others, by purinergic receptors (Schwab et al., 2005; Lister et

al., 2007; Ulmann et al., 2008; Marcillo et al., 2012) and Toll-Like Receptors (Kigerl et al., 2007). Consistently, we observed, in thoracic cord segments just below (T9-T11) and above (T6-T8) the transection, a long lasting (up to 60 days post-surgery) increase in the expression of mRNAs encoding P2XA, P2X7 and TLR4 receptors.

Numerous reports in the literature ascribe to activated microglia an important role in neuropathic pain (see Tsuda et al., 2013, for a review), notably in pain consecutive to spinal cord injury (Carlton et al., 2009; Kim et al., 2013), and the marked induction of OX-42 mRNA in SCT rats is congruent with these data. In fact, IL-6, IL1-ß, and TNF-α can induce, by themselves, central (spinal) sensitization, thus maintaining neuropathic pain (Peng et al., 2006; Chi et al., 2008; Chen et al., 2011; Guptarak et al., 2013; Liu et al., 2013). The huge induction of IL-6 and IL1-ß that occurred on day 2 post-surgery suggests that these cytokines were involved more in the induction than in the maintenance of SCT-evoked neuropathic pain. In contrast, TNF-α would be more concerned by pain maintenance as SCT-induced up-regulation of its transcript in spinal T6-T8 segments was as pronounced at day 60 as at day 2 post-surgery. The strong increase in IL-10 mRNA that occurred shortly after the lesion might be linked to some inhibitory control of neuropathic pain for the first days after SCT, through the anti-inflammatory potency of IL-10 (Karam et al., 2007; Genovese et al., 2009) and/or its neuroprotective effects in spinal cord injured models (Brewer et al., 1999; Zhou et al., 2009). Overall, in contrast to that found for at least some of them in spinal tissues, none of the 10 genes studied were up-regulated in dorsal root ganglia above the lesion beyond two weeks post-surgery, supporting the idea that SCT-induced neuropathic pain did not involve some peripheral hypersensitivity but really corresponded to central neuropathic pain, as already emphasized above.

Within the spinal cord, GFAP mRNA up-regulation after SCT was delayed compared to that of transcripts encoding the pro-inflammatory cytokines IL-1ß, IL-6 and TNF-α, in line with the production and release of these cytokines resulting from *primary* microglial activation (John et al., 2003) and *secondary* induction of astrogliosis after injury (Cernak et al., 2005; Rohl et al., 2007; Tian et al., 2007). That astrogliosis with an up-regulation of GFAP (Nesic et al., 2005; Gwak et al., 2012) – like that found in SCT rats - contributes to neuropathic pain after spinal cord injury (by contusion or hemisection) is supported by the fact that blockade of GFAP or connexin 43 (specifically expressed in astrocytes) reduced pain in spinal cord-lesioned rats (Cronin et al., 2008; Gwak et al., 2008).

In addition to its cardinal role in neurogenesis and maintenance of neuronal functions in peripheral and central nervous systems (Isakson, 1995; Montaño et al., 2010), BDNF is known to be implicated in neural injury-induced neuropathic pain (Wang et al., 2009). Indeed, direct intrathecal injection produces neuropathic pain-like behavioral reactions (Constandil et al., 2012) and BDNF up-regulation at spinal

level has been shown to be causally related to neural injury-induced pain (Zhou et al., 2010). Accordingly, in SCT rats, the increased expression of BDNF mRNA in DRG might be related to a hyperexcitability of nociceptors via a down regulation of BK channel activity (Cao et al., 2012). On the other hand, BDNF mRNA down regulation in the spinal cord of SCT rats might be prejudicial for the regeneration of tissues and indirectly contribute to long lasting ongoing neuronal and glial damage (Koda et al., 2002).

Conclusion

Spinal cord transection at thoracic level in rats appeared to generate a highly reproducible model of at-level neuropathic pain of central origin, suitable for pharmacological studies aimed at testing innovative treatments targeted specifically on central neuropathic pain.

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Legends to figures

Figure 1 : Time-course changes in the pressure threshold to trigger hindlimb withdrawal in spinal cord-transected rats

Pressure threshold values were determined using a graded series of von Frey filaments applied onto hindpaw. Each point is the mean + S.E.M. of independent determinations in 6 rats. « Cut-off SCT » corresponded to the maximal pressure tested in operated rats ; even at this high pressure level (100 g), no response of hindlimbs was evoked for the first 9 days post-surgery. The « Threshold sham » corresponded to the minimal pressure (60 g) to which sham-operated animals start to respond by hindlimb withdrawal.

** P < 0.01, *** P < 0.01, significantly different from 100 g « cut-off SCT » value. One-way ANOVA for repeated measures (effect of a drug over time) followed by a Dunnett's test.

Figure 2 : Body territories with increased mechanical sensitivity in spinal cord-transected rats

Pressure threshold values to trigger nocifensive responses were determined using a graded series of von Frey filaments applied throughout the body. Comparison with sham-operated rats (C) showed that pressure threshold values differed in SCT rats only in a limited territory (6 cm^2) located rostrally to the spinal cord section (at T8-T9, horizontal bar with arrow heads) and in hindpaws (black areas), where reactions were obtained for pressure values significantly less than in controls. Time course (day 2 to day 60) changes in spinal cord transected rats showed that supersensitivity (allodynia) in the at-level area rostral to the lesion was already detected at day 2 (D2) post-surgery, then extended and increased up to a plateau reached at D14 post-surgery. At hindpaw level, supersensitivity developed much later (from D21 post-surgery). Data were obtained in 8-14 rats at each time.

Figure 3 : Time-course changes in nocifensive reactions to von Frey filaments application in the « at-level » allodynic territory rostral to the lesion in spinal cord-transected rats

Pressure threshold values to trigger biting (of the filament), shaking or escape were determined using a graded series of von Frey filaments applied onto the allodynic at-level area on the back at various times (in days) after surgery (0 on abscissa). Each bar is the mean + S.E.M. of independent determinations in 8 rats.

*** P < 0.001 compared to control (intact) rats (C on abscissa). One-way ANOVA for repeated measures (effect of a drug over time) followed by a Dunnett's test.

Figure 4 : Anti-allodynic effects of acute administration of morphine (A), tapentadol (B), ketamine (C) or baclofen (D) in spinal cord-transected rats

Acute administration of morphine (1, 3 or 10 mg/kg s.c.), tapentadol (10 or 20 mg/kg i.p.), ketamine (50 mg/kg i.p.), baclofen (10 mg/kg i.p.) or their respective vehicle was performed (0 on abscissa, arrow) in rats whose spinal cord had been transected at T8-T9 level one month before. Pressure threshold values to trigger nocifensive biting were determined using von Frey filaments applied within the at-level allodynic territory at various times after treatment. Each point is the mean + S.E.M. of independent determinations in n rats.

C on abscissa : Control (naive) rats (prior to surgery).

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared to respective values in vehicle-treated rats.

One-way ANOVA for repeated measures (effect of a drug over time) followed by a Dunnett's test.

Figure 5 : Time-course changes in tissue levels of transcripts encoding ATF3 (A), OX42 (B) or GFAP (C) in dorsal root ganglia and spinal cord at various times after spinal cord transection

Real-time quantitative RT-PCR determinations were made in T6-T8 and T9-T11 dorsal root ganglia, T6-T8 and T9-T11 spinal cord segments and the cervical and lumbar enlargements at various times (in days, abscissa) after spinal cord transection at T8-T9 level. Data are expressed as the ratio of specific mRNA over GAPDH mRNA [R.Q.(A.U.)]. Each bar is the mean + S.E.M. of 6-12 independent determinations. Sham values at every postoperative time are pooled into one condition referred as C (control).

* $P < 0.05$, * $P < 0.01$, *** $P < 0.001$ compared to control levels in sham-operated rats.Two-way ANOVA followed by Bonferroni test.

Figure 6 : Short- and long-term changes in levels of transcripts encoding IL-6 (A), IL-1ß (B), TNF-α (C) and IL-10 in dorsal root ganglia and spinal tissues in spinal cord-transected rats

Real-time quantitative RT-PCR determinations were made in T6-T8 and T9-T11 dorsal root ganglia and T6-T8 and T9-T11 spinal segments at day 2, 15 or 60 (abscissa) after spinal cord transection at T8-T9 level. Data are expressed as the ratio of specific mRNA over GAPDH mRNA [R.Q.(A.U.)]. Each bar is the mean + S.E.M. of 6-12 independent determinations. Sham values at every postoperative time are pooled into one condition referred as C (control).

* P < 0.05, * P < 0.01, *** P < 0.001 compared to control levels in sham-operated rats Two-way ANOVA followed by Bonferroni test.

Figure 7 : Short- and long-term changes in levels of transcripts encoding P2X4 (A), P2X7 (B) and TLR4 (C) in dorsal root ganglia and spinal tissues in spinal cord-transected rats

Real-time quantitative RT-PCR determinations were made in T6-T8 and T9-T11 dorsal root ganglia and T6-T8 and T9-T11 spinal segments at day 2 or 60 (abscissa) after spinal cord transection at T8-T9 level. Data are expressed as the ratio of specific mRNA over GAPDH mRNA [R.Q.(A.U.)]. Each bar is the mean + S.E.M. of 6-12 independent determinations. Sham values at every postoperative time are pooled into one condition referred as C (control).

** P < 0.01, *** P < 0.001 compared to control levels in sham-operated rats. Two-way ANOVA followed by Bonferroni test.

Figure 1

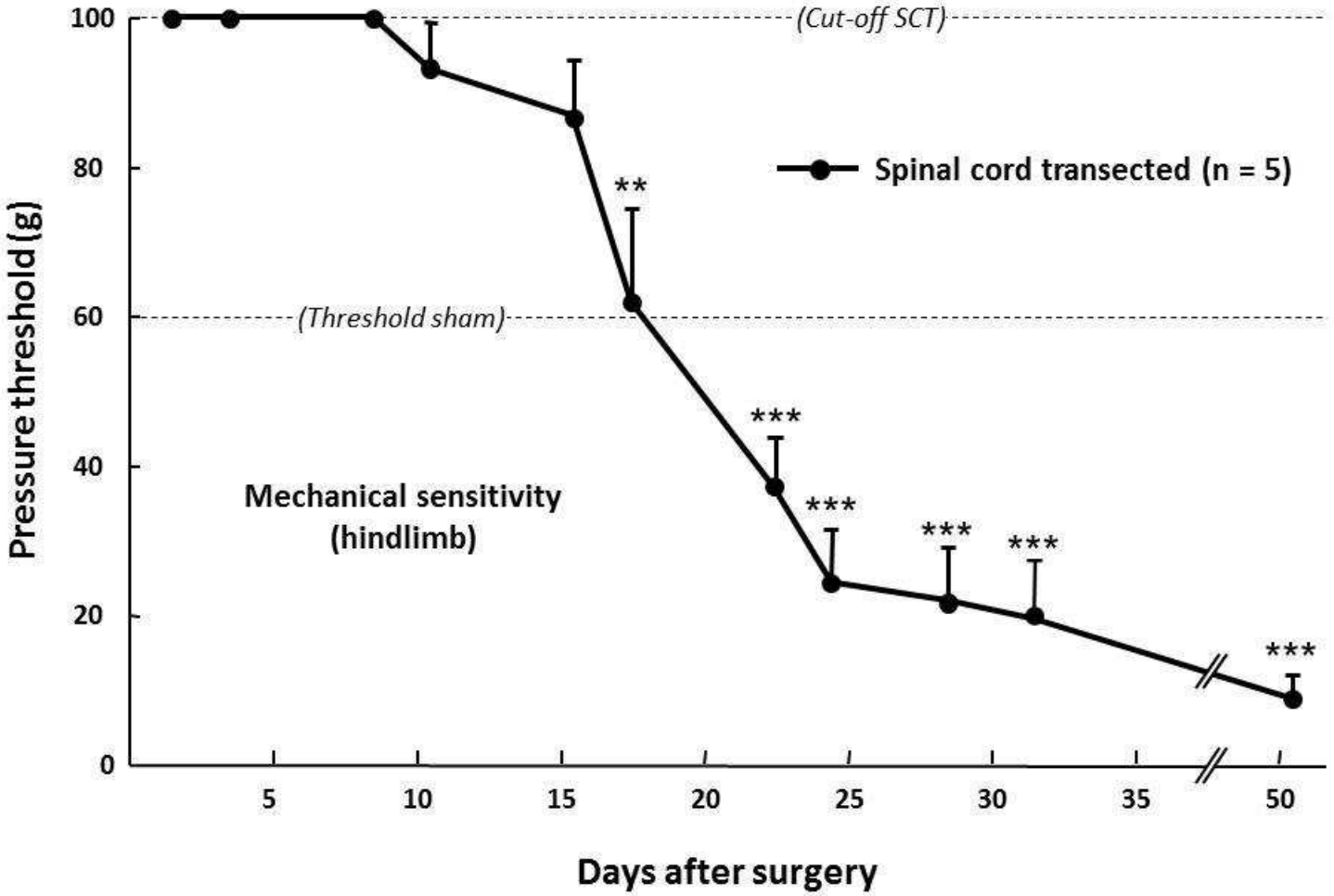
100
80
60
40
20
0
(Cut-off SCT)
(Threshold sham)
Spinal cord transected (n = 5)
**
***
***
***
***
***
Mechanical sensitivity
(hindlimb)
5
10
15
20
25
30
35
50
Pressure threshold (g)
Days after surgery

Figure 2

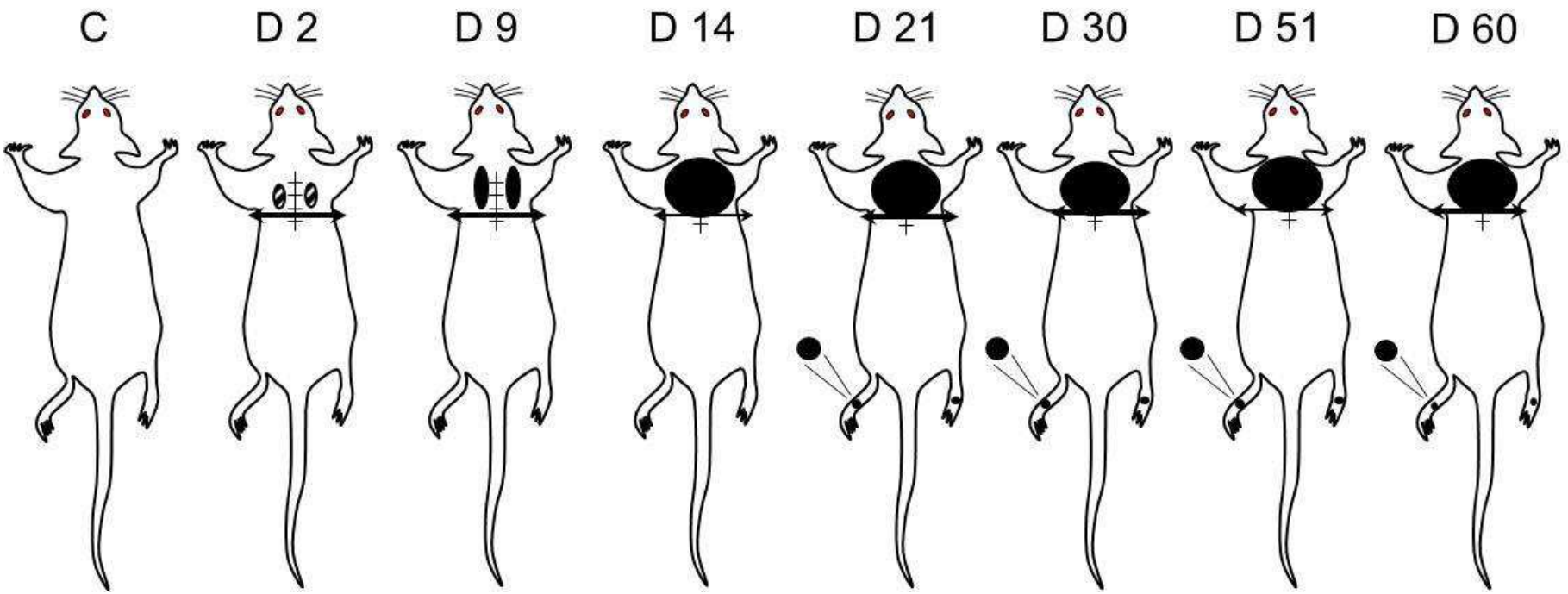

Figure 3

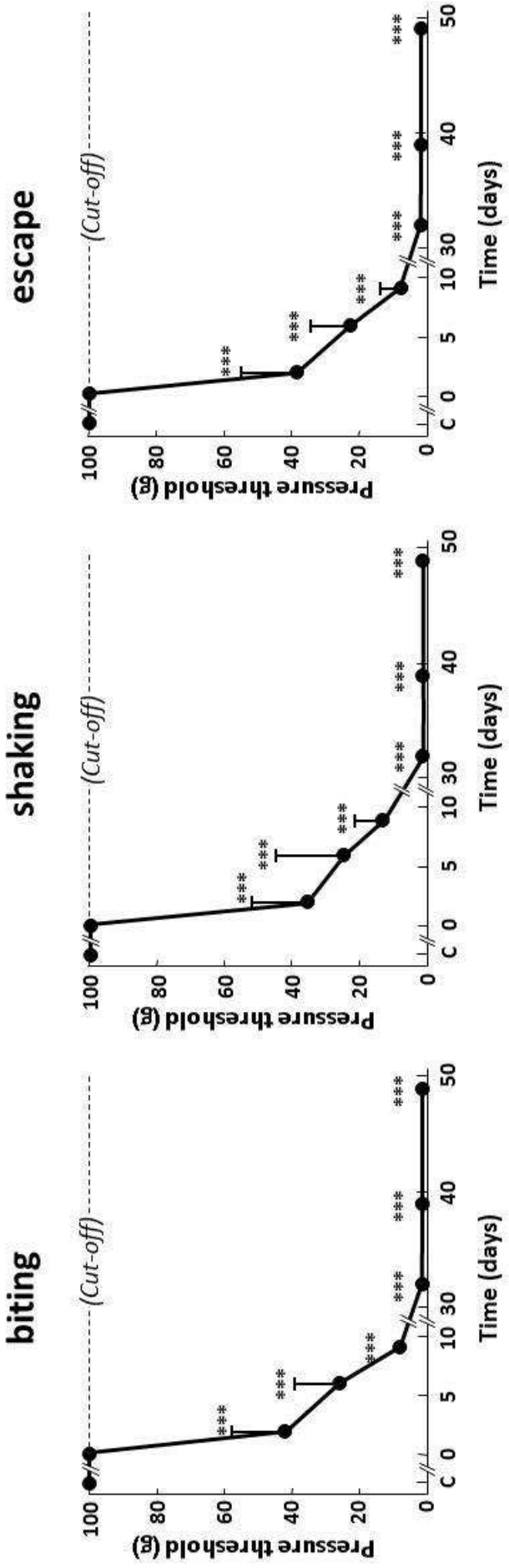
biting
shaking
escape
(Cut-off)
Pressure threshold (g)
Time (days)
C
0
5
10
30
40
50
***

Figure 4

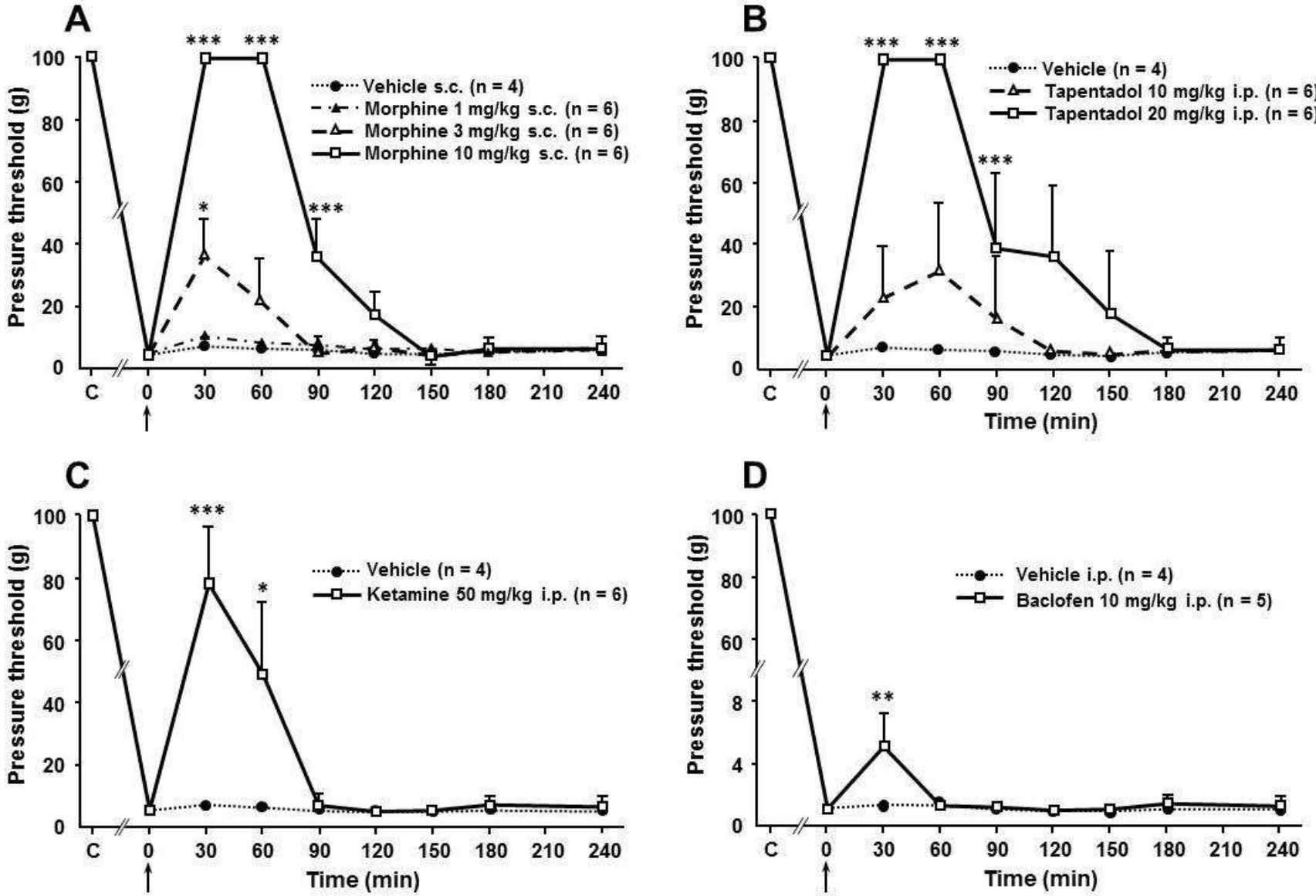
A
B
C
D
Pressure threshold (g)
Time (min)
Vehicle s.c. (n = 4)
Morphine 1 mg/kg s.c. (n = 6)
Morphine 3 mg/kg s.c. (n = 6)
Morphine 10 mg/kg s.c. (n = 6)
Vehicle (n = 4)
Tapentadol 10 mg/kg i.p. (n = 6)
Tapentadol 20 mg/kg i.p. (n = 6)
Ketamine 50 mg/kg i.p. (n = 6)
Vehicle i.p. (n = 4)
Baclofen 10 mg/kg i.p. (n = 5)
C
0
30
60
90
120
150
180
210
240

Figure 5

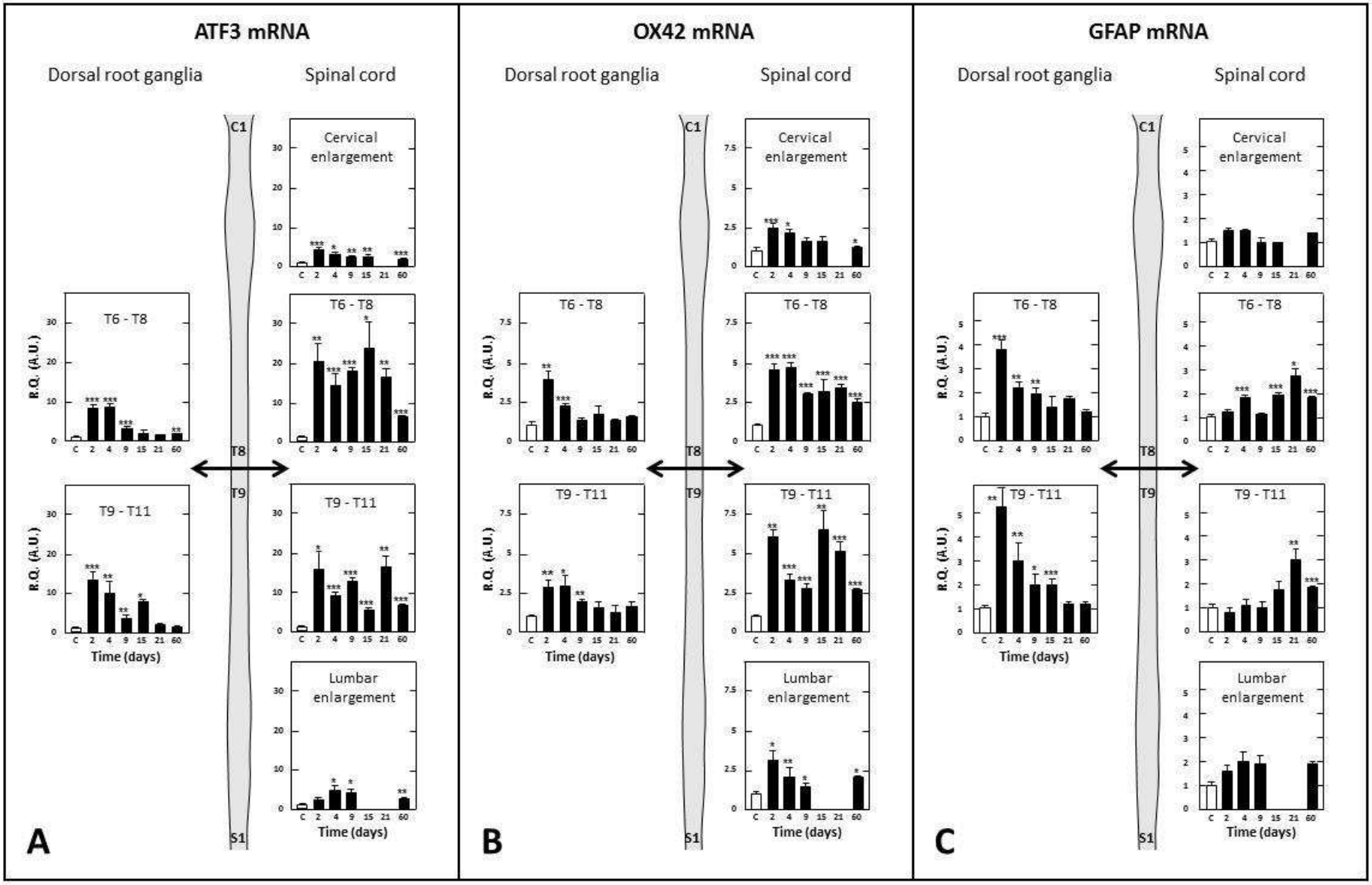
ATF3 mRNA
OX42 mRNA
GFAP mRNA
Dorsal root ganglia
Spinal cord
C1
T8
T9
S1
T6 - T8
T9 - T11
Cervical enlargement
Lumbar enlargement
R.Q. (A.U.)
Time (days)
A
B
C

Figure 6

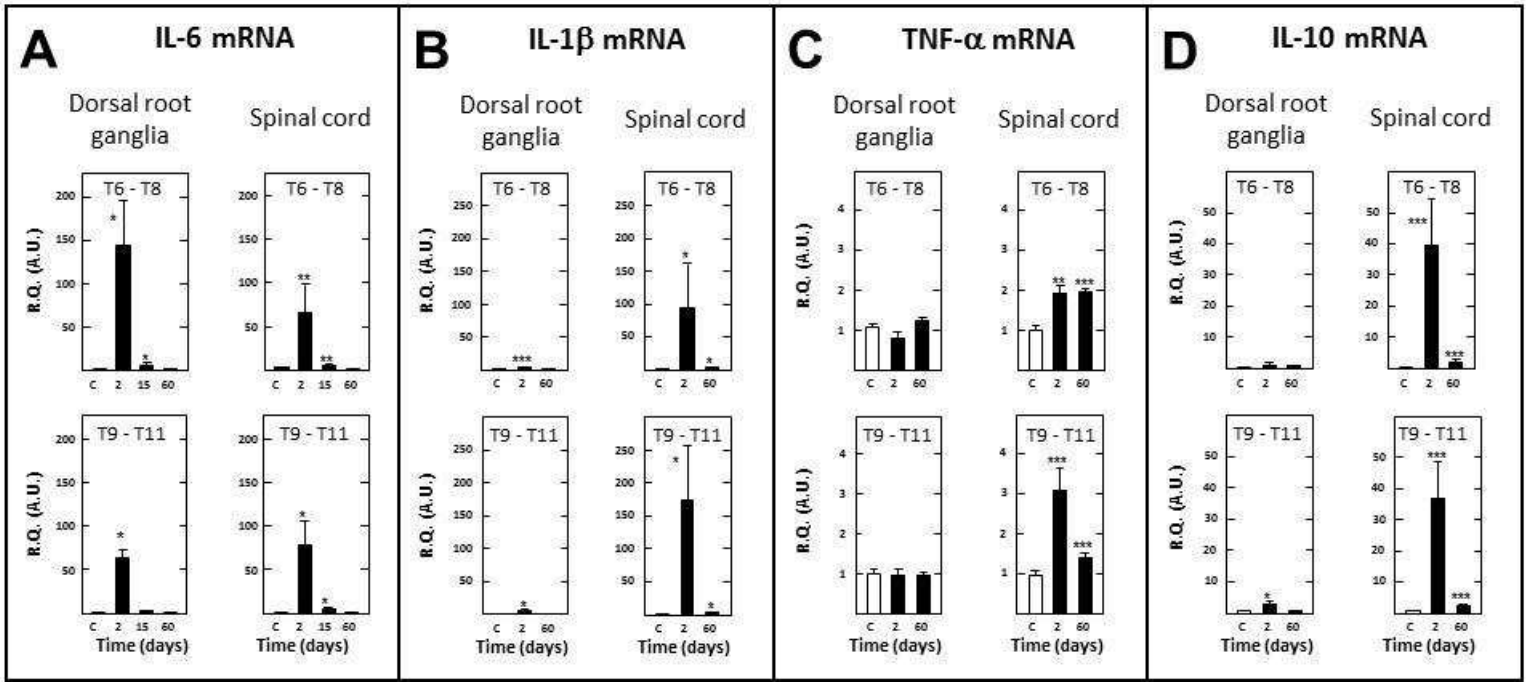
A
IL-6 mRNA
B
IL-1β mRNA
C
TNF-α mRNA
D
IL-10 mRNA
Dorsal root ganglia
Spinal cord
T6 - T8
T9 - T11
R.Q. (A.U.)
C 2 15 60
C 2 60
Time (days)

Figure 7

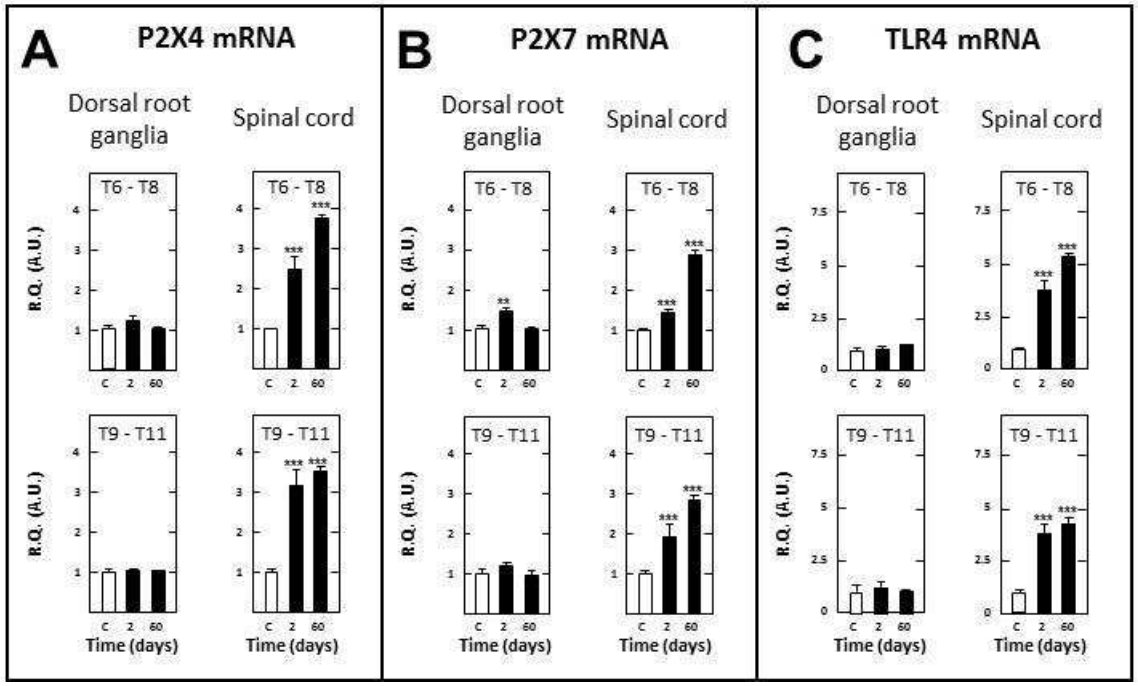
A
P2X4 mRNA
Dorsal root ganglia
Spinal cord
T6 - T8
T9 - T11
R.Q. (A.U.)
C 2 60
Time (days)
B
P2X7 mRNA
Dorsal root ganglia
Spinal cord
T6 - T8
T9 - T11
R.Q. (A.U.)
C 2 60
Time (days)
C
TLR4 mRNA
Dorsal root ganglia
Spinal cord
T6 - T8
T9 - T11
R.Q. (A.U.)
C 2 60
Time (days)

Table 1: Pharmacological treatments tested for potential anti-allodynic effects in spinal cord-transected rats

Drugs	Pharmacological effect	Dose	Efficacy on biting behavior
Morphine	Opioid receptor agonist	1, 3, 10 mg/kg s.c.	+++
Tapentadol	Opioid receptor agonist and noradrenaline reuptake inhibitor	10, 20 mg/kg i.p.	+++
Ketamine	NMDA receptor antagonist	50 mg/kg i.p.	++
Baclofen	GABAB receptor agonist	10 mg/kg i.p.	+
Clonazepam	Benzodiazepine (agonist)	0.25, 2 mg/kg i.p.	-
Gabapentin	Blockade of calcium channel α2δ subunit	30, 100, 300 mg/kg i.p.	-
Pregabalin	Blockade of calcium channel α2δ subunit	30 mg/kg i.p.	
Amitriptyline	Tricyclic antidepressant	10 mg/kg i.p.	-
Amitriptyline + Gabapentin	Tricyclic antidepressant + Blockade of calcium channel α2δ subunit	10 mg/kg i.p. + 100 mg/kg i.p.	-
Cyclotraxin B	TrkB receptor blocker	20 mg/kg i.p.	-
Naratriptan	5-$HT_{1B/D}$ receptor agonist	0.1 mg/kg i.p.	-
Ondansetron	5-HT_3 receptor antagonist	20 µg i.t.	-
8-OH-DPAT	5-$HT_{1A/7}$ receptor agonist	0.25 mg/kg i.p.	-

+++ : potent anti-allodynic effect (complete recovery of control mechanical sensitivity) ; ++ : potent but short lasting anti-allodynic effect ;

+ : modest but significant anti-allodynic effect ; - : inactive treatment.

ARTICLE 2

Caractérisation comportementale, pharmacologique, biochimique et immunohistochimique de la douleur neuropathique suite à une administration intrathécale de BDNF chez le rat - Comparaison avec la douleur neuropathique provoquée par la ligature du nerf sciatique.

I - INTRODUCTION

Du fait de la résistance des douleurs neuropathiques aux antalgiques classiques, il est d'usage de recourir à d'autres classes pharmacologiques telles que des antidépresseurs ou des anticonvulsivants (Baud, 2007). Cependant, leur efficacité modeste - le plus souvent - et les effets secondaires qu'ils engendrent sont des limites à leur utilisation. Mieux caractériser les douleurs neuropathiques et élucider les mécanismes clefs mis en jeu sont des préalables nécessaires en vue de développer des traitements plus spécifiques et plus efficaces. De nombreuses données de la littérature montrent que le BDNF a un rôle pro-nociceptif dans la modulation de la douleur. Ainsi, son expression au niveau de la moelle épinière est augmentée chez les rats rendus neuropathiques par la ligature du nerf sciatique, et l'administration intrathécale d'anticorps ou de ligands spécifiques pour bloquer les voies de signalisation en aval du récepteur TrkB du BDNF permet de réduire les douleurs neuropathiques (Coull et al., 2005; Zhao et al., 2006; Constandil et al., 2012). Au contraire, l'injection intrathécale ou intracisternale de BDNF induit des phénomènes d'hyperalgésie et d'allodynie de longue durée chez le rat (Yajima et al., 2005; Constandil et al., 2012). Nous avons mis à profit ces observations pour proposer un nouveau modèle de douleur neuropathique : le modèle «BDNF it » consistant en l'injection intrathécale de BDNF (3 ng) chez le rat.

Nous avons caractérisé ce modèle au niveau comportemental par des tests de nociception validés (filaments de von Frey, test de Randall-Selitto). De plus, nous avons analysé par RT PCR et immunohistochimie l'expression de gènes associés à la neuroinflammation et à l'activation microgliale afin de voir si ces phénomènes interviennent chez les rats BDNF i.t. (comme c'est le cas dans d'autres modèles de douleurs neuropathiques). En effet, l'expression du marqueur de souffrance neuronale ATF3 ainsi que celles des marqueurs d'activation microgliale Iba-1 et OX-42 sont fortement augmentées chez des rats souffrant de douleurs neuropathiques d'origine

centrale ou périphérique, et le blocage de leur expression diminue les manifestations comportementales d'hyperalgésie et d'allodynie (Latrémolière et al., 2008; Carlton et al., 2009 ; Tsuda et al., 2013). Comme P38 est une protéine particulièrement impliquée dans l'activation de la microglie et que, de plus, son rôle dans l'induction de douleurs neuropathiques a été démontré (Jin et al., 2003 ; Tsuda et al., 2004 ; Hains and Waxman, 2006 ; Ji et al., 2007), nous l'avons également étudié, comme les marqueurs précédents, dans le modèle BDNF it. Enfin, nous avons recherché si notre modèle était « prédictif » en ce qui concerne la pharmacologie. A cette fin, nous avons analysé les effets de l'administration d'agents pharmacologiques efficaces chez l'homme et dans d'autres modèles animaux de douleurs neuropathiques, notamment la prégabaline, la duloxetine, le clonazepam ou encore l'agomélatine.

II - RESULTATS

II.1. - Induction de l'hyperalgésie et de l'allodynie chez les rats BDNF it – Comparaison avec les rats CCI-SN

La ligature du nerf sciatique (CCI-SN) conduit à l'apparition d'une hyperalgésie et d'une allodynie au niveau de la patte ipsilatérale au nerf ligaturé, dont l'intensité est maximale à partir de deux semaines après la pose des ligatures. Grâce au test de Randall et Selitto, on peut alors observer une diminution des seuils de pression qu'il convient d'appliquer pour provoquer le retrait de la patte (-23,4%, $P<0,05$) puis la vocalisation (-30,8%, $P<0,01$).

L'injection i.t. de BDNF provoque aussi l'apparition d'une hyperalgésie et d'une allodynie au niveau des pattes postérieures, dont l'intensité est maximale à partir de 4-5 jours après l'injection. Dans le test de Randall et Sellito, on peut observer une diminution des seuils de pression pour lesquels le rat retire sa patte (-33,8%, $P<0,05$), puis vocalise (-35,9%, $P<0,01$).

Ces effets sont intégralement reproduits par l'agoniste du récepteur TrkB, le MIML4-11, également injecté par voie intrathécale à la dose de 1 µg. Ainsi, dans le test de Randall-Selitto, on note une nette diminution du seuil de pression pour lequel le rat vocalise (-28,4%) 10 jours après l'injection i.t. de ce composé (Figure 19).

En revanche aussi bien chez les rats BDNF i.t. que chez les rats CCI-SN, aucun signe de douleur neuropathique n'est décelable au niveau céphalique. Dans les deux cas, l'hyperalgésie et l'allodynie ne concernent que le territoire extra-céphalique.

II.2 - Quantification d'ARNm spécifiques par RT-qPCR

Nous avons quantifié l'expression de différents facteurs au niveau de la moelle lombaire (L4-L5) chez les rats CCI-SN et BDNF i.t. (24 h et 10 jours après l'injection) en comparaison avec les témoins correspondants.

Chez les rats CCI-SN, la ligature entraine une augmentation importante des taux tissulaires des transcrits ATF3 (x 8,57, P<0,01), OX-42 (x10,2, P<0,01) et IL6 (x 4,57, P<0,05).

En revanche, l'injection i.t. de BDNF n'a pas induit de variation notable des taux des transcrits IL6 et ATF3, mais a provoqué une diminution des taux de l'ARNm OX-42 qui est significative au 10^{e} jour post-injection (-44,4%, P<0,05).

Par ailleurs, la ligature du nerf sciatique induit aussi une augmentation significative des taux tissulaires des transcrits BDNF (x 1,62, P<0,05) et NR2B (x 4,2, P<0,05) au 15^{e} jour après la chirurgie.

L'injection i.t. de BDNF entraine aussi une augmentation significative (x1,48, P<0,05) des taux d'ARNm BDNF au 10^{e} jour post-injection. En revanche, les taux des transcrits NR2B ne présentent aucune variation aux deux temps considérés après l'injection it de BDNF.

II.3 - Immunomarquages

Nous avons réalisé les immunomarquages de deux protéines témoins de l'activation microgliale, Iba1 et P-p38.

- *Iba1*

Du côté ipsilatéral à la ligature du nerf sciatique, on constate une augmentation significative du marquage Iba1 chez les animaux CCI-SN par rapport aux *sham* (+22,8 % ± 5,3, P< 0,001) et aux animaux naïfs (+32,5% ± 7.0 P<0,001). Au contraire, chez les rats BDNF i.t., le nombre de cellules marquées par les anticorps Iba1 est faiblement mais significativement diminué en comparaison des animaux « véhicule » (-11,2% ± 4,4, P<0,05).

- *P-p38*

Dans les couches 1-2 de la moelle dorsale, on constate une augmentation significative du marquage P-p38 chez les rats CCI-SN, en comparaison des rats naïfs et *sham*, des 2 côtés, ipsilatéral (+64.02 % ± 5,4) et contralatéral (+26,9 % ± 8,7) à la ligature. On note également une augmentation du marquage P-p38 chez les rats *sham* comparés aux rats naïfs du côté ipsilatéral (+ 54,1% ± 5,4) indiquant que la chirurgie suffit, par elle-même, à induire une activation microgliale.

Chez les rats BDNF i.t. non stimulés, nous n'avons pas détecté de modification significative du marquage P-p38 par rapport à celui quantifié chez les rats injectés avec le véhicule. En revanche, une stimulation mécanique appliquée sur la patte arrière droite 24 h avant le sacrifice de l'animal induit une augmentation significative du marquage P-p38 au niveau de la corne dorsale, aussi bien du côté ipsilatéral (+56,56% ± 16,00 pour les rats BDNF i.t. stimulés versus les rats BDNF it non stimulés, et + 47,83% ± 16,01 pour les rats BDNF i.t. stimulés versus les rats véhicule i.t. stimulés ; $P<0,01$) que du côté contralatéral (+46,39 % ± 12,60 pour les rats BDNF i.t. stimulés versus les rats BDNF i.t. non stimulés, $P<0,01$) à la stimulation (Figure 20).

II.4 – Pharmacologie

Nous avons poursuivi notre étude comparative en évaluant l'impact de différents agents pharmacologiques sur l'hyperalgésie chez des rats CCI-SN d'une part, et chez des rats BDNF i.t. d'autre part. Les composés administrés ont été notamment des antidépresseurs et des anti-épileptiques, communément utilisés en clinique pour soulager les douleurs neuropathiques (Baud, 2007). De plus, disposant d'un antagoniste du récepteur TrkB du BDNF, la cyclotraxine B (Cazorla et al., 2010), nous avons évalué son potentiel anti-hyperalgésique aussi bien chez les rats BDNF i.t. que chez les rats CCI-SN.

- *Cyclotraxine B*

Chez le rat BDNF i.t., la cyclotraxine B injectée en i.p. à la dose de 20 mg/kg réduit l'hyperalgésie induite par le BDNF. On constate en effet une augmentation du seuil de pression nécessaire pour déclencher la vocalisation dans le test de Randall et Selitto. La réduction de l'effet du BDNF est d'environ 50%, 5 jours après l'injection. Chez le rat CCI-SN, l'effet est plus marqué puisque la cyclotraxine B supprime presque totalement l'hyperalgésie induite par les ligatures. Cet effet est significatif 5 jours après l'injection i.p. de cyclotraxine B.

- *Agomélatine, clonazepam, duloxétine, prégabaline, tapentadol*

Les différents agents pharmacologiques testés ont une efficacité semblable dans les deux modèles CCI-SN et BDNF i.t.

Ainsi, la prégabaline, le tapentadol et l'agomélatine suppriment totalement l'hyperalgésie de manière transitoire dans les deux modèles tandis qu'au contraire, le clonazepam et la duloxétine ne modifient pas le seuil de réaction de l'animal dans le test de Randall et Selitto.

III - DISCUSSION

Dans le modèle « BDNF i.t. », l'hyperalgésie et l'allodynie sont de même intensité que chez les rats CCI-SN. Nous montrons ici que le BDNF a un rôle non seulement dans l'induction de la douleur neuropathique mais également dans son maintien, puisque 15 jours après la ligature du nerf sciatique, ou 10 jours après l'injection i.t. de BDNF, l'administration systémique de cyclotraxine B, à une dose qui bloque le récepteur TrkB du BDNF *in vivo* dans le tissu nerveux central (Cazorla et al., 2010), réduit les comportements de type douloureux chez le rat. De plus, l'effet anti-hyperalgésique de la cyclotraxine B persiste pendant au moins 5 jours après son administration, montrant clairement l'impact bénéfique du blocage de la voie BDNF/TrkB pour soulager les douleurs neuropathiques. Il peut paraître surprenant qu'un traitement aigu avec la cyclotraxine B ait un effet aussi long. Néanmoins, le blocage par la cyclotraxine B du phénomène de LTP qui peut être induit par le BDNF (Zhou et al., 2008) dans la corne dorsale de la moelle pourrait être l'une des composantes majeures de l'action anti-hyperalgésique de cet antagoniste de TrkB. Enfin, l'injection intrathécale d'un agoniste sélectif du récepteur TkB, le MIML4-11 (Fig. 19), est suffisante pour induire une hyperalgésie équivalente à celle observée chez le rat BDNF i.t. et chez le rat CCI-SN, confirmant ainsi que l'induction de l'hyperalgésie par le BDNF passe par le récepteur TrkB.

Le modèle BDNF i.t. se différencie du modèle CCI-SN du fait qu'il n'induit pas de souffrance neuronale ni de neuroinflammation comme le montre l'absence d'induction d'ATF3 ou de cytokines proinflammatoires. De plus, on n'observe pas d'activation microgliale ni d'activation de la MAPK p38 chez les rats BDNF i.t. Au contraire, une légère baisse de l'expression des marqueurs microgliaux Iba1 et OX-42 a même été observée chez ces animaux. Il semble donc que ni l'induction de cytokines pro-inflammatoires, ni l'activation microgliale et de la MAPK p38 ne soient nécessaires à l'induction de l'hyperalgésie et/ou de l'allodynie. Néanmoins, Zhou et al. (2010) ont rapporté une activation de la microglie spinale par le BDNF chez le rat. Une activation microgliale transitoire pourrait donc intervenir après l'injection i.t. du facteur neurotrophique. Dans ce contexte, il serait intéressant d'évaluer l'action d'un bloquant de l'activation microgliale comme la minocycline sur l'hyperalgésie induite par l'injection i.t. de BDNF.

Quoi qu'il en soit, l'injection intrathécale de BDNF entraine une sensibilisation des mécanismes activateurs de p38 dans la moelle épinière puisqu'une stimulation mécanique soutenue (mais non douloureuse) au niveau d'une patte postérieure a provoqué une augmentation de la densité des

cellules immunoréactives Pp38+ dans la corne dorsale (ipsi et contra-latérale) au niveau lombaire chez les rats BDNF it (mais pas chez les rats contrôles saline i.t.) (Fig. 20).
La persistance de l'hyperalgésie jusqu'à au moins 30 jours après une injection unique de BDNF i.t. pourrait s'expliquer par une induction du gène BDNF. D'ailleurs, Saarelainen et al. (2001) ont rapporté que le BDNF pouvait exercer un rétrocontrôle positif sur sa propre synthèse, dans l'hippocampe chez la souris. Il serait intéressant de déterminer la source principale de production du BDNF dans le modèle BDNF i.t. (expériences de co-marquages Iba1-BDNF, GFAP-BDNF, et NeuN-BDNF). D'autre part, comme l'activation de TrkB conduit à la phosphorylation de la sous unité NR2B des récepteurs NMDA, il serait également intéressant d'étudier la cinétique de ce processus dans les neurones de la corne dorsale de la moelle lombaire chez les rats BDNF i.t. Enfin, l'étude de l'adressage membranaire des récepteurs NMDA mériterait aussi d'être conduite chez les rats BDNF i.t.

IV – CONCLUSION

En résumé, le modèle BDNF i.t. a montré tout son intérêt pour l'étude des douleurs neuropathiques chez le rat. De fait, il est beaucoup plus facile à mettre en œuvre qu'une ligature de nerf puisqu'il ne nécessite qu'une seule injection intrathécale sous anesthésie légère. De plus, il répond aux agents pharmacologiques connus pour réduire les douleurs neuropathiques chez l'homme et dans d'autres modèles validés de ce type de douleurs. En revanche, il n'implique pas d'activation microgliale ni de réaction inflammatoire, sans doute du fait que le BDNF induit les mécanismes sous-tendant l'hyperalgésie et l'allodynie en aval de l'intervention de ces phénomènes déclenchés par une lésion neurale. L'efficacité de traitements antalgiques dans le modèle BDNF i.t. permet donc de préciser qu'ils agissent en aval de l'activation microgliale et de la neuroinflammation. En l'occurrence, l'efficacité avérée de la prégabaline, du tapentadol et de l'agomélatine laisse à penser qu'une éventuelle action anti-inflammatoire de ces composés (Jang et al., 2012 ; Molteni et al., 2013) n'est pas impliquée dans leurs effets anti-hyperalgésiques.

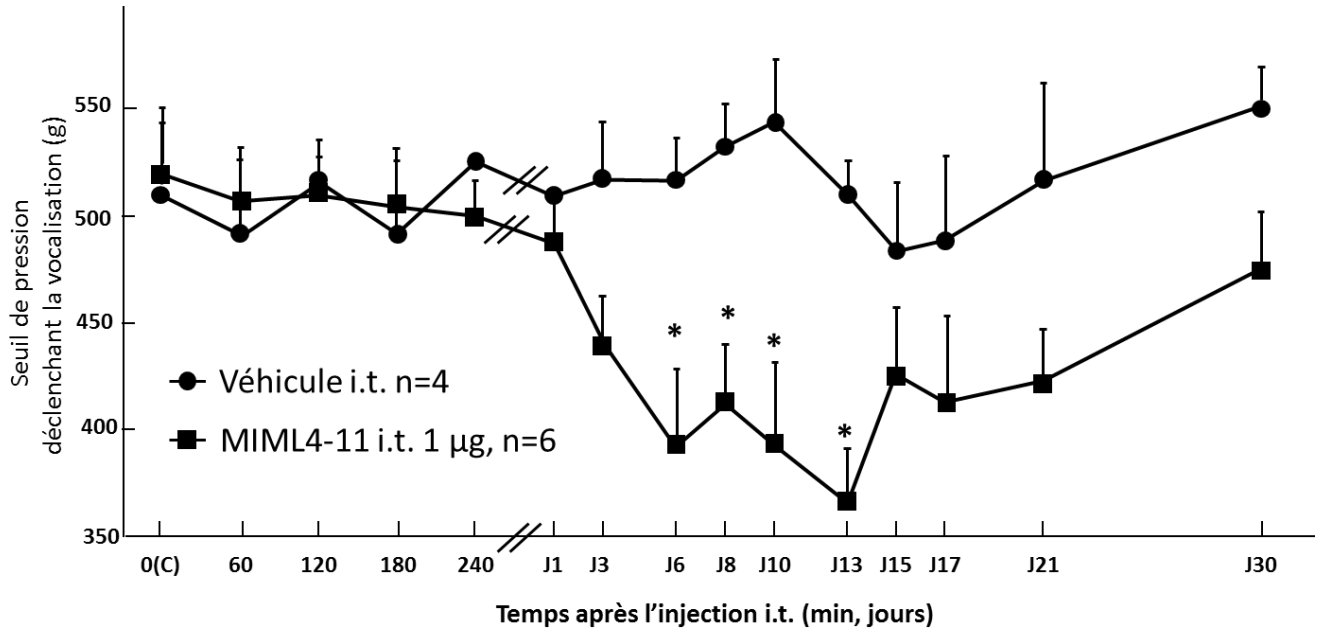

Figure 19 : Effet hyperalgésique de l'injection i.t. de l'agoniste des récepteurs TrkB, MIML4-11, chez le rat naïf.

L'injection i.t. de MIML4-11 (1µg/rat) ou de son véhicule (NaCl à 0,9%) a été effectuée au temps 0 chez des rats naïfs, et le seuil de pression appliquée à la patte postérieure pour déclencher la vocalisation (test de Randall et Selitto) a été déterminé à des temps variés (abscisse) après le traitement.

Chaque point est la moyenne + E.S.M de n déterminations indépendantes.

* P< 0,05 par rapport à la valeur correspondante chez les rats injectés avec le véhicule (ANOVA à 2 voies, test de Bonferroni).

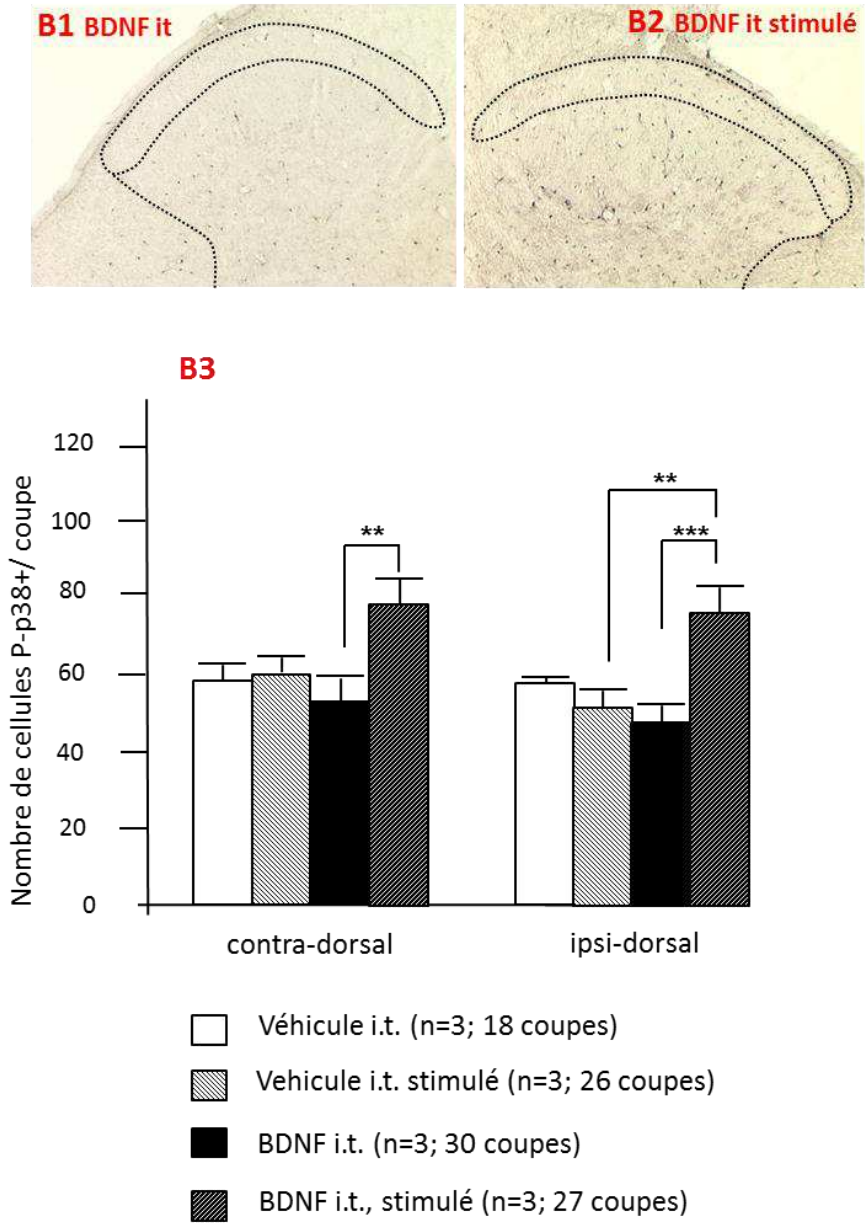

Figure 20 : Effets d'une stimulation mécanique non nociceptive sur l'expression de P-p38 dans les couches superficielles de la corne dorsale de la moelle épinière (renflement lombaire) chez des rats prétraités par le BDNF (3 ng, voie i.t.) ou son véhicule (12 µL NaCl à 0,9%, voie i.t.).
La stimulation unilatérale d'une patte postérieure a été effectuée (cf p 76) 10 jours après l'administration i.t. de BDNF ou de son véhicule. L'immunomarquage de P-p38 au niveau des couches I et II (zone délimitée en B1 et B2) montre une augmentation de la densité des cellules P-p38+ chez les rats BDNF i.t. « stimulés » (B3).
** P< 0,01, *** p<0,001 par rapport à la valeur correspondante chez les rats BDNF non stimulés (ANOVA à 1 voie, test de Newman-Keuls).

Behavioral, pharmacological, biochemical and immunohistochemical characterization of neuropathic-like pain evoked be intrathecal administration of BDNF in rats – Comparison with neuropathic-like pain caused by sciatic nerve ligation

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ABSTRACT

A large body of evidence has shown that Brain Derived Neurotrophic Factor (BDNF), which is induced by nerve lesion, plays a key role in the resulting neuropathic pain. Indeed, intrathecal (i.t.) administration of BDNF itself was found to cause hyperalgesia and allodynia in intact healthy rats. In the present study, we investigated whether neuropathic-like pain symptoms induced by i.t. BDNF could be used as a model to assess the anti-hyperalgesic effects of various drugs, in comparison with the well validated model that consists of chronic constriction injury (CCI) to the sciatic nerve (SN).

Experiments were performed in adult male Sprague-Dawley rats. Rats were either treated acutely with BDNF (3.0 ng i.t.) or its vehicle (0.9% NaCl) under slight isoflurane anesthesia, or subjected to unilateral CCI-SN under pentobarbital anesthesia. Animals were then treated acutely by various drugs and subjected to the Randall-Selitto test to assess hyperalgesia at hindpaw level. Immunostaining of marker proteins and RT-qPCR of specific transcripts were performed in dorsal root ganglia and spinal cord.

A long lasting mechanical hyperalgesia was induced by acute i.t. BDNF in intact healthy rats. At their maximum, i.e. 4-9 days after i.t. BDNF, the resulting decreases in pressure threshold values to trigger nocifensive responses were of the same amplitude as those found two weeks after surgery in CCI-SN rats. Systemic pretreatment with cyclotraxin B, to block BDNF-TrkB receptor, completely prevented mechanical hyperalgesia normally evoked by i.t. BDNF. Furthermore, cyclotraxin B also partially reversed BDNF-evoked mechanical hyperalgesia. and reduced neuropathic-like pain in rats which underwent CCI-SN two weeks before. Acute treatments with the anticonvulsant pregabalin (30 mg/kg i.p.), the opioid antalgic tapentadol (10 mg/kg i.p.) or the antidepressant agomelatine (100 mg/kg i.p.) transiently reversed mechanical hyperalgesia in both i.t. BDNF injected- and CCI-SN lesioned-rats. However marked differences also exist between both models because immunohistochemistry and RT-qPCR investigations showed that microglial markers (Iba1, OX42, P-38p) were induced in CCI-SN rats but not BDNF i.t. rats. These data support the idea that BDNF i.t. rats would be especially appropriate to investigate the potential anti-hyperalgesic effects of drugs acting downstream of neural lesion-induced neuroinflammation processes.

Key words: BDNF, neuropathic pain, chronic constriction injury, microglia activation, Trk B receptor

I - INTRODUCTION

Neuropathic pain is a major health problem because of its resistance to most classical analgesic drugs. To date, treatments aimed at alleviating neuropathic pain are mainly based on empirical data with drugs of various therapeutic classes such as antidepressants and anticonvulsants which have generally only a modest efficacy and can induce undesirable side effects (Jensen et al., 2009).

Despite significant progress in the knowledge of physiopathological mechanisms underlying neuropathic pain in animal models (Ji and Suter, 2007; Basbaum et al., 2009; Ji et al., 2013), very few innovative treatments have emerged for the last thirty years. The aim with animal models is to reproduce as much as possible the human pathology, so as to predict the potential efficacy of pharmacological compounds to be used as drugs in the clinics. In the pain field, numerous animal models of neuropathic pain have been developed which most often consist of lesioning a dorsal root (Chung and Chung, 2001) or a peripheral nerve (by ligation, compression or section), in particular the infraorbital nerve, the sciatic nerve or one of its branches (Wall et al., 1979; Bennett and Xie, 1988; Vos et al., 1994; Decostered and Wall, 2000). However, the reliability of these models for the identification of new drugs of therapeutic value in humans is limited, and several examples in the recent past illustrated that treatments effective in animal models were ineffective in patients (Sindrup et al., 2006; Landry et al., 2012).

In spite of these limitations, all models allowed the clear-cut demonstration that neuroinflammatory processes and microglial activation play key roles in physiopathological mechanisms underlying neuropathic pain. In particular, prevention of microglia activation by drugs such as minocycline was found to significantly reduce allodynia and hyperalgesia evoked by nerve lesion (Latremolière et al., 2008). In addition, specific blockade of microglia activation markers such as the MAP kinase p38 (Ji and Suter, 2007), which is markedly enhanced upon neuroinflammation induced by peripheral nerve lesion (Kumar et al., 2003), reduces neuropathic pain in rats. Among factors produced and released by activated microglial cells, Brain Derived Neurotrophic Factor (BDNF) has a key position regarding neuropathic pain evoked by neural lesion (Merighi et al., 2008), notably because it is, on its own, strongly algogenic, producing both hyperalgesia and allodynia when administered directly at the spinal or brain stem level (Yajima et al., 2005; Constandil et al., 2011, 2012). As acute intrathecal injection of BDNF avoids surgical interventions to cause neural lesion, we investigated whether this easy treatment would allow us to set-up a novel, reliable and reproducible, animal model

of neuropathic-like pain. Accordingly, we compared, in rats, the time course onset of mechanical hyperalgesia and allodynia after intrathecal injection of BDNF (BDNF i.t.) with that induced by unilateral chronic constriction injury to the sciatic nerve (CCI-SN). In order to validate the BDNF i.t. model, we further assessed whether BDNF-induced hyperalgesia could be alleviated by drugs known to be effective in patients suffering from neuropathic pain and in CCI-SN rats. Finally, we used quantitative RT-PCR and immunohistochemical approaches to investigate whether or not microglial and neuroplasticity markers were induced in the spinal cord of BDNF i.t. rats like that found in CCI-SN rats (Jergovà and Cizkovà, 2007; Latrémolière et al., 2008).

II – MATERIALS AND METHODS

II.1 - Animals

Adult male Sprague-Dawley rats (175-200 g on arrival) were purchased from Charles River Breeding Center (L'Arbresle, France). They were housed in a controlled environment (22°± 1 °C, 60% relative humidity, 12:12 h light-dark cycle, lights on at 7:00 a.m.) with food and water available ad libitum. Animals were allowed to habituate to these housing facilities without any handling for at least 1 week before experiments. In all cases, experiments were performed in conformity with the Guidelines of the Committee for Research and Ethical Issues of the International Association for the Study of Pain (IASP; Zimmermann, 1983) and strictly followed the Institutional Guidelines that are in compliance with national and international laws and policies for use of animals in neuroscience research (Council Directive 87-848, October 19, 1987, Ministère de l'Agriculture et de la Forêt, Service vétérinaire de la santé et de la protection animale, permissions nb 75 116 to M.H., nb 006228 to S.B., nb A752128 to S.M).

II.2 - Surgery

II.2.1 - Chronic constriction injury to the sciatic nerve

Rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.), and unilateral CCI-SN was made according to the procedure of Bennett and Xie (1988). Briefly, the right common sciatic nerve was exposed at mid-thigh level by blunt dissection through the biceps femoris muscle. Proximal to the trifurcation, 10 mm of nerve was freed from adhering tissue and four loose

ligatures (5.0 silk suture) were tied loosely around the sciatic nerve (1 mm spacing). To obtain the desired degree of constriction, the criterion formulated by Bennett and Xie (1988) was applied: the ligatures reduced the diameter of the nerve by a just noticeable amount, and retarded, but did not interrupt, the epineurial circulation. Finally, muscle and skin were closed in layers with silk suture (4.0). Animals were allowed to recover for one week in their home cage (3 rats in 40 x 40 cm^2, 18 cm high).

II.2.2 - Intrathecal injection

Rats were slightly anesthetized using isoflurane (3% in air) and injected into the subarachnoid space between the L5 and L6 vertebrae using a 26 G needle connected to a Hamilton syringe (Mestre et al., 1994). Each rat received 12 µL of saline (controls) or of a BDNF (3 ng, "BDNF i.t.", Preprotech, 92200 Neuilly, France) solution in saline. Mechanical sensitivity tests (see II.3) were then performed at various times starting on the following day.

II.3 – Behavioral tests

II.3.1 - Paw pressure test

Mechanical nociceptive thresholds, expressed as grams (g), were measured using an Ugo Basile algesimeter (Bioseb, 92370 Chaville, France) according to Randall and Selitto (1957). An increasing pressure was applied onto the nerve-injured hindpaw of CCI-SN rats and both hindpaws in BDNF i.t. rats until paw withdrawal and then vocalization (i.e. a squeak when the paw was maintained under pressure) were obtained. As paw withdrawal is a spinally coordinated reflex whereas vocalization is a supraspinal integrated response (Le Bars et al., 2001), this test can provide some indirect indications on the respective contributions of spinal versus supraspinal mechanisms in the modulatory effects of drugs on nociceptive responses. All tests were conducted between 10 a.m. and 6 p.m. in a quiet room where rats acclimatized for at least one hour before test performance. Basal responses were established on the day before nerve injury or intrathecal injection. On day 14 after surgery for CCI-SN rats, and on day 10 for BDNF i.t. rats, when hypersensitivity to mechanical stimulation had fully developed (see Results), responses thresholds were measured again to establish post-injury baseline, and pharmacological treatments started immediately thereafter. Only animals showing clear-cut

mechanical hypersensitivity (approximately 60% of CCI-SN rats and 80% of BDNF i.t. rats), with a reduction of nociceptive thresholds of about 30% compared to sham/vehicle rats, were used for pharmacological studies. Mechanical nociceptive thresholds were evaluated at regular time intervals (30 or 60 min) after drug administration by an experienced person blind to treatment groups.

II.3.2 – von Frey filaments test

Each rat was placed under a Plexiglass box (31 x 19.5 x 14 cm) positioned on a wire grid platform (5 mm x 5 mm mesh) through which von Frey filaments were applied for 2-3 sec to the lateral plantar surface of hindpaw. The minimal force filament for which animals responded with either a brisk paw withdrawal and/or an escape attempt allowed determination of the mechanical response threshold. When no response was observed, the force of the thickest filament (60 g) was arbitrarily assigned as the withdrawal threshold (see Latrémolière et al., 2008). As above, this test was carried out by an experienced person blind to treatment groups.

II.4 - Pharmacological treatments

Cyclotraxin B (Bio S&T, Montreal, Canada), pregabalin (Sequoia, Pangbourne, UK), tapentadol (Grünenthal, Aachen, Germany), agomelatine (Servier, Suresnes, France), duloxetine (Eli Lilly, Indianapolis, USA), amitriptyline (Roche, Basel, Switzerland) and clonazepam (Roche) were administered using routes and doses reported to be active at respective molecular targets in relevant literature (see refs cited in the sections of Results and Discussion).

All the drugs were injected i.p. in a volume of 1 mL/kg. Tapentadol, cyclotraxin B and pregabalin were dissolved in 0.9 % NaCl. Duloxetine and agomelatine were dissolved in 1% hydroxyethylcellulose (HEC) in water. Clonazepam was dissolved in a mixture of 50% water:50% ethanol. Control animals received respective vehicles using the same routes of administration.

II.5 - Immunohistochemical labeling

Rats were deeply anesthetized with pentobarbital (50 mg/kg i.p.) and perfused through the heart with a heparin (25 IU/ml)-saline solution (during 4 min), followed by 0.12 M phosphate buffered saline (PBS, pH 7.4) containing 4% paraformaldehyde, 0.1% glutaraldehyde and 0.05%

picric acid (during 24 min), and finally by 20% sucrose solution (during 5 min). The spinal cord was removed and cryoprotected in 20% sucrose solution overnight. Coronal floating sections (30 µm thick) of the lumbar enlargement were collected in containers filled with 0.02 M PBS 0.02M containing 0.02% sodium azide, and kept in this medium overnight at room temperature. All sections were then processed in parallel, with the same reagents, under the very same conditions.

To label phospho-P38 and Iba 1 protein, sections were incubated with the respective primary antibody (rabbit anti-phospho P38, 1/3,000 , Cell signaling Inc; rabbit anti Iba1, 1/8000, Wako chemicals Inc), diluted in PBS-T-azide (0.02 M PBS containing 0.3% Triton X-100 and 0.02% sodium azide) overnight at room temperature. After rinsing for 30 min with PBS, the sections were incubated for 1 h with the secondary antibody (biotinylated goat anti-rabbit, 1/1000, Vector, Burlingame, CA, USA) in PBS containing 0.3% Triton X-100. After rinsing again for 30 min with PBS, the sections were finally incubated for 1 h in the avidin-biotin horseradish-peroxidase solution (ABC Vectastain kit Elite, Vector). They were then rinsed with PBS (15 min), and immunolabeling was revealed with peroxidase substrate kit (SK-4100, Vector) according to the manufacturer's instructions. Sections were sequentially treated with 50%, 70%, 90%, 95% absolute alcohol and finally xylene, and cover-slipped with Eukitt (Sigma-Aldrich, St Louis, MO, USA).

Immunoreactive cells were observed under brightfield illumination in 30 µm thick coronal sections every 180 µm along lumbar enlargement of the spinal cord. We took into account all labeled cells without considering the staining intensity. Iba 1 and phospho-P38 immunoreactive cells within the superficial laminae (laminae I and II) of the dorsal horn at L4-L5 levels were counted using the software Mercator. Special care was taken to count immunoreactive cells within a strictly similar area in the different groups of rats. Digitized photomicrographs were made using a CCD color video camera, connected to a microscope, which sent an RGB (Red, Green, Blue) output to a Macintosh computer. Images at different focal planes were captured and digitized as 24 bit color-scale using Openlab software (Improvision, Coventry, UK). An operator allowed the combination, pixel-by-pixel, of images in different focal planes. These operations resulted in the production of one image by incorporating the darker value of the corresponding pixel in each focal plane for each of red, green and blue color plans. Images were exported to Adobe-Photoshop (version 6.0) in order to mount adjacent digitized images for making a final high resolution and large field image. Then brightness, contrast and image scale

were adjusted. Finally, additional indications and anatomical landmarks were incorporated to the final figure as described in detail elsewhere (Kayser et al., 2010b).

II.6 – Real time quantitative RT-PCR measurements

BDNF i.t. rats (24 h and 10 days after the injection), CCI-SN rats (14 days after the surgery) and their respective controls were killed by decapitation. The dorsal halves of the lumbar cord (ipsilateral quadrant for CCI-SN) at L4-L6 were rapidly dissected at 0-4 °C, and immediately frozen in liquid nitrogen to be stored at -80 °C. Total RNA was extracted using the NucleoSpin RNA II extraction kit (Macherey-Nagel, 67722 Hoerdt, France) and quantified using a NanoDrop. First-stranded cDNA synthesis (from 660 ng total RNA per 20 µl of reaction mixture) was carried out using High capacity cDNA reverse transcription kit (Applied Biosystems, Courtaboeuf, France). PCR amplification, in triplicate for each sample, was performed using ABI Prism 7300 (Applied Biosystems), TaqMan® Universal PCR Master Mix No AmpErase® UNG (Applied Biosystems) and Assays-on-Demand Gene Expression probes (Applied Biosystems) for target genes: ATF3 (assay ID Rn00563784_m1), OX42 (Rn00709342_m1), IL6 (Rn00561420_m1), BDNF exon IX (Rn02531967_s1) and NR2B (Rn00680474_m1) subunit of NMDA receptors. Semi-quantitative determinations were made with reference to the reporter gene encoding glyceraldehyde 3-phosphate dehydrogenase (GaPDH; Rn99999916_s1). The polymerase activation step at 95°C for 15 min was followed by 40 cycles of 15 s at 95°C and 60 s at 60°C. The validity of the results was checked by running appropriate negative controls (replacement of cDNA by water for PCR amplification; omission of reverse transcriptase for cDNA synthesis). Specific mRNA levels were calculated after normalizing from GaDPH mRNA in each sample. Data are presented as relative mRNA units compared to control values (see Latrémolière et al., 2008).

II.7 - Statistical analyses

All values are expressed as mean ± S.E.M. Areas under the time-course curves (AUC) were calculated using the trapezoidal rule. For behavioral tests, results were analyzed using two-way ANOVA with factor treatment and repeated measures over time. For RT-PCR data, the $2^{-\Delta\Delta Ct}$ method (Schmittgen and Livak, 2008) was used to analyze the relative changes in specific mRNA levels. For immunohistochemistry and qRT-PCR data, the Student's t test was used to compare

treated versus control group when only 2 groups were analysed. A one way ANOVA followed by a Newman Keuls test was used when comparison was made between 3 or more groups. In all cases, significance level was set at $P < 0.05$.

III - RESULTS

III. 1 – Time course development of mechanical hyperalgesia and allodynia in CCI-SN and BDNF i.t. rats.

Sciatic nerve ligation induced mechanical hyperalgesia at the ipsilateral hindpaw with a maximum intensity two weeks after the surgery (Fig. 1 A). At this time, CCI-SN rats showed significant decreases in both paw withdrawal threshold value (-23.4 % $P<0.05$) and vocalization threshold value (-30.8%, $P<0.01$) determined using the Randall-Selitto test (Fig. 2).
Intrathecal administration of BDNF (3 ng) also induced mechanical hyperalgesia, but more rapidly than CCI-SN, with a maximal decrease in paw threshold values reached as soon as one week post injection (Fig. 1B). As shown in figure 2, intensity of mechanical hyperalgesia as assessed from decreases in hindpaw withdrawal and vocalization threshold values was similar in BDNF i.t. and CCI-SN rats.
Von Frey filaments test showed that mechanical allodynia also appeared after chronic constriction injury to the sciatic nerve (Fig. 1C) and intrathecal administration of BDNF (Fig. 1D). As already noted about hyperalgesia, allodynia was as pronounced in BDNF i.t. rats as in CCI-SN rats, but it reached its maximum much earlier in the former than the latter model (Fig. 1D compared to Fig. 1C).

III.2 - Effects of various drugs on mechanical hyperalgesia in BDNF i.t. rats compared to CCI-SN rats

III.2.1 – Effects of pregabalin, tapentadol, agomelatine, duloxetine and clonazepam

Two weeks after sciatic nerve ligation in CCI-SN rats or 10 days after i.t. injection of BDNF in BDNF i.t. rats, acute treatment with pregabalin (30 mg/kg i.p.) significantly reduced hyperalgesia. As illustrated in Figs. 3A and 3B, similar time courses of the drug effect were observed in both models, with complete reversal of mechanical hyperalgesia at 2-3 hours post-treatment.

In both CCI-SN and BDNF i.t. rats, i.p. injection of tapentadol (10 mg/kg) also reduced mechanical hyperalgesia as shown by significant increases in pressure threshold values to trigger vocalization in the Randall-Selitto test (Figs 3C, 3D). However, these effects are of shorter duration compared to pregabalin, and only partial reversal of hyperalgesia was noted in BDNF i.t. rats.

As shown in figures 3E and 3F, the antidepressant agomelatine (45 mg/kg, i.p.) significantly reduced mechanical hyperalgesia in both CCI-SN rats and BDNF i.t. rats. As previously noted for tapentadol, these effects were transient since partial recovery of control level of pressure threshold values was observed only 30 and/or 60 min after treatment.

In contrast with the former three drugs, the antidepressants amitriptyline (10 mg/kg i.p.) and duloxetine (10 mg/kg i.p.) and the benzodiazepine clonazepam (0.25 mg/kg i.p.) did not significantly affect pressure threshold values at any time up to three hours post-treatment in both CCI-SN and BDNF i.t. rats (not shown).

III.2.2 – Effects of TrkB receptor blockade by cyclotraxin B

In CCI-SN rats, acute treatment with cyclotraxin B (20 mg/kg i.p.) induced a progressive increase in pressure threshold value to trigger vocalization in the Randal-Selitto test. As illustrated in figures 4A,C, this effect was long lasting since it was still significant 20 days after treatment. Similarly, acute administration of cyclotraxin B at the same dose increased the pressure threshold value to trigger vocalization in BDNF i.t. rats (Figs 4B,D). This effect was of lower duration than in CCI-SN rats since it reached statistical significance 5 days after administration of the TrkB receptor antagonist, but was no longer detected at later times (Fig. 4B).

III.3 – qRT-PCR determinations of neuroinflammation/microglial activation markers in the spinal cord of CCI-SN versus BDNF i.t. rats

In CCI-SN rats, a marked upregulation of transcripts encoding ATF3 (x 8.57, $P < 0.01$), OX-42 (x 10.2, $P < 0.01$) and IL-6 (x 4.57, $P < 0.05$) was observed in the ipsilateral dorsal half of the lumbar enlargement of the spinal cord two weeks after the surgery (Fig. 5A). By contrast, i.t. BDNF injection did not induce such variations in spinal levels of mRNAs encoding ATF3 and IL-

6, and even a down regulation of OX42 mRNA (-44.4 %, $P < 0.05$) was detected on the 10th day post BDNF treatment, when hyperalgesia and allodynia reached their maximal intensities (see Fig. 1).

Moreover, sciatic nerve ligation induced significant upregulation of both BDNF (x 1.62, $P < 0.05$) and NR2B (x 4.2, $P < 0.05$) mRNA levels in the ipsilateral dorsal half of the lumbar enlargement of the spinal cord 15 days after the surgery (Fig. 6A). Similarly, BDNF mRNA upregulation was observed in the same spinal area (but bilaterally) in BDNF i.t. rats (x 1.48, $P < 0.05$) 10 days, but not two days, post-injection (Fig. 6B). In contrast, NR2B mRNA levels did not change in BDNF i.t. rats compared to respective controls (Fig. 6B).

III.4 – Immunohistochemical labeling of microglial activation markers Iba1 and phospho-P38 at spinal level in CCI-SN versus BDNF i.t. rats

III.4.1 - Iba1 immunolabeling

A significant increase of Iba1 immunostaining was observed in the ipsilateral superficial layers of the dorsal horn at L5-L6 level two weeks after the surgery in CCI-SN rats compared to sham rats (+ 22.8% ± 5.3, $P < 0.001$) and to naïve animals (+ 32.5% ± 7.0, $P < 0.001$). On the contrary, in BDNF i.t. rats, the density of Iba1 immunoreactive cells in the very same spinal cord area was slightly, but significantly, decreased (-11.2% ± 4.4, $P<0.05$)
as compared to saline i.t. rats (Figs 7A, 7B).

III.4.2 – Phospho-p38 immunolabeling

At L5-L6 level of the lumbar enlargement of the spinal cord, within laminae I and II of the dorsal horn ipsilateral to the surgery, we noted a significant increase of phospho-p38 immunostaining in CCI-SN rats compared to naïve and sham rats (Figs. 7A, 7B). A significant increase was also noted within the contralateral laminae I and II (+ 26.9 % ± 8.7, CCI-SN vs sham, $P < 0.01$, not shown), but of lower amplitude than that observed within ipsilateral laminae (+ 64.0% ± 11.8, CCI-SN vs sham, Fig. 7B, $P < 0.01$). In addition, we also detected a significant difference in phospho-p38 immunostaining in sham-operated rats compared to naïve animals at the ipsilateral side (+ 54.1% ± 12.6, Fig. 7B, $P < 0.05$), indicating that surgery on its own activated microglia.

In sharp contrast with that found in CCI-SN rats, no significant modification of phospho-p38 immunostaining could be detected in BDNF i.t. rats compared to saline i.t. rats (Figs 7A,7B).

IV - DISCUSSION

Because of their resistance to classical antalgics, other pharmacological classes of drugs such as antidepressants and anticonvulsants are commonly used to treat neuropathic pain in patients (Baud, 2007). However, these drugs have a rather modest efficacy, and their undesirable side effects limit their use. Therefore, better characterization of neuropathic pain and its underlying mechanisms are essential to develop more efficient and better tolerated new treatments. With this purpose, our laboratory worked until now on the CCI-SN model of neuropathic pain, which has the advantage to be well characterized (Latrémolière et al., 2008). However, this model has some limits, in particular the inflammation induced by the surgery and the long delay, about two weeks, before clear-cut development of typical neuropathic pain-like behavior in rats. Numerous physiopathological mechanisms are activated in this model and it is relatively poorly adapted to the study of specific cellular/molecular steps likely to contribute to the neuropathic pain physiopathology. This led us to assess the potential interest of this new model of neuropathic pain that consists of acute intrathecal administration of a subnanomolar dose (3 ng) of the trophic factor BDNF. We showed here that in this BDNF i.t. model, the rat developed, within only 5 days, hyperalgesic-like and allodynic-like behaviors as severe as after CCI-SN without any surgery. Knowing the critical role of BDNF in the neuropathic pain processes (Garraway et al., 2003; Geng et al., 2010), the validation of this model would allow in-depth study of the downstream molecular pathways involved in hyperalgesia and allodynia, and eventual identification of new molecular target(s) of potential therapeutic interest.

According to convergent data, BDNF plays a major role in hyperalgesia induction (Geng et al., 2010). Here, we demonstrated that BDNF has also a role in the maintenance of neuropathic pain because 15 days after chronic constriction injury to the sciatic nerve, or 10 days after BDNF i.t. injection, systemic administration of cyclotraxin B, at a dose blocking TrkB receptor of BDNF in the central nervous system in vivo (Cazorla et al., 2010), reduced nociceptive behavior in the rat. Moreover, this cyclotraxin B-anti-hyperalgesic effect lasted for at least 5 days after its administration, showing the major efficacy of blocking BDNF/TrkB pathway to relieve

neuropathic pain. Such a long lasting effect is surprising. Actually, this TrkB antagonist probably acts at different levels of the nociceptive and integrative pain pathways. In particular, it has been shown that LTP is implicated in the synaptic sensitization underlying hyperalgesic processes (Zhang et al., 2004) and the blockade by cyclotraxin B of this phenomenon (which can be induced by BDNF, see Zhou et al., 2008) in spinal dorsal horn could be of high relevance regarding the anti hyperalgesic effect of this TrkB antagonist. Recently, Constandil et al. (2012) showed that a single systemic administration of cyclotraxin B could also reduce for several days the allodynia induced by ligation of the infraorbital nerve or by intracisternal injection of BDNF, showing that the effects we observed in case of extracephalic pain (spinal system) also occur for cephalic pain (trigeminal system). However, the hypothesis that cyclotraxin B acts as an LTP inhibitor at the level of the spinal dorsal horn and the trigeminal caudalis nucleus (like that demonstrated in the hippocampus by Cazorla et al., 2010) to explain the capacity of this TrkB antagonist to reduce neuropathic pain would have to be tested in complementary studies.

After confirming the cardinal role of BDNF in neuropathic pain processes, we started the characterization of the BDNF i.t. model. First, we aimed at investigating whether BDNF could induce the same physiopathological mechanisms as sciatic nerve ligation.

In the CCI-SN model, sciatic nerve ligation induces a neural injury which triggers, through ATP release, microglial activation and, in turn, cytokines and BDNF release which are responsible for central sensitization (Woolf, 2004; Latrémolière et al., 2008). Nerve lesion-induced microglia activation demonstrated to play a key role in the induction of neuropathic pain (Echeverry et al., 2008). Indeed, administration of minocycline, a microglial inhibitor, prevents or delays neuropathic pain development after nerve lesion (Ledeboer et al., 2005; Latrémolière et al., 2008). Knowing the preponderant role of microglia, it was interesting in our BDNF i.t. model to look for a possible microglial activation correlated with hyperalgesia triggered by this neurotrophic factor. For this aim, we measured by qRT-PCR the mRNA levels of the marker of microglial activation OX-42 and by immunohistochemistry the number of cells expressing two other microglial activation markers Iba1 and p38.

Our results indicated that BDNF did not induce microglial activation but, on the contrary, a slight decrease in both OX-42 mRNA levels and the number of immunolabeled Iba 1 cells could be observed, without any P-p38 activation, 10 days after i.t. injection of BDNF. Therefore, it can be inferred that microglial and p38 MAPK activation were not necessary for the induction of

hyperalgesia. This absence of microglial activation could reflect the absence of tissue injury and nerve lesion in BDNF i.t. model (indeed, there was no upregulation of ATF3 mRNA). This is in agreement with previously reported studies. Thus, i.t. ATP injection, which produces a downstream release of BDNF and nociceptive behavior, does not induce p38 activation (Nakagawa et al., 2008). Nevertheless, other authors (Zhou et al., 2010) showed that a transient microglial activation could be induced by BDNF, an effect which would have been no longer detectable under our experimental condition. In this context, It would be of interest to investigate whether microglia inactivation by minocycline can affect hyperalgesia induced by i.t BDNF.

Numerous studies demonstrated that peripheral nerve lesion induces a sustained p38 activation that contributes to neuropathic pain syndrome (Jin et al., 2003; Svensson et al., 2003). Indeed, p38 inhibitor administration can prevent the induction of neuropathic pain (Jin et al., 2003). In line with these data, although we could not detect any P-p38 activation under resting conditions, we confirmed that BDNF can sensitize mechanisms underlying p38 activation in the spinal cord because a sustained unilateral mechanical (non nociceptive) stimulation of the hindlimb could induce a bilateral increase of P-p38+ cell density within the dorsal horn of the lumbar cord in i.t. BDNF treated rats (unpublished data).

In CCI-SN rats, microglial activation induces the release of pro-inflammatory cytokines such as IL-6 and IL-1β which are upregulated in the lumbar spinal cord after nerve lesion (Lee et al., 2004; Latrémolière et al., 2008). Minocycline can prevent the upregulation of cytokines and associated neuropathic pain suggesting the existence of a causal link between both phenomena (Latrémolière et al., 2008). In contrast, i.t. BDNF administration did not produce any upregulation of transcripts encoding IL-6 further confirming the absence of microglial activation. However, other cytokines such as IL-1β and TNF-α have to be investigated before any firm conclusion could be made.

The only factor upregulated in BDNF i.t. rats was BDNF itself, in line with previous in vitro and in vivo studies showing that this neurotrophic factor can promote its own synthesis (Saarelainen et al., 2001; Wilbrand et al., 2006; Yasuda et al., 2007; Zheng et al 2012). However it remains to be establish how BDNF could be upregulated since microglia, usually considered as the major cellular phenotype responsible for BDNF production in models of nerve lesion (Woolf 2004), was not upregulated in BDNF i.t. rats. Actually, BDNF can be upregulated in small

and medium dorsal root ganglia neurons (Pezet et al., 2002) as well as in astrocytes after spinal cord injury (Dougherty et al 2000); Double immunolabelling with BDNF antibodies and with either Iba1, GFAP or Neun antibodies would allow the identification of cell phenotype(s) with upregulated BDNF in BDNF i.t. rats.

It has been clearly established that BDNF can increase synaptic efficacy in an NMDA-dependent way through PKC and PKMζ activation in spinal dorsal horn (Garraway et al., 2003; Melemedjian et al., 2013). Moreover, BDNF can regulate the expression and the traffic of NMDA receptors in hippocampal neurons in culture (Caldeira et al., 2007). On the other hand, i.t. injection of an NMDA receptor antagonist can reduce allodynia induced by i.t. BDNF injection (Geng et al., 2010). As NR2B transcripts were not upregulated 10 days after BDNF i.t. injection when hyperalgesia was maximum it can be inferred that BDNF did not induce NR2B transcription but promoted its activation via phosphorylation, as previously reported in cortical and hippocampal neurons (Lin et al., 1998). Direct investigations of NR2B phosphorylation in spinal tissues will have to be performed after BDNF i.t. injection.

Further investigations should also concern AMPA receptors and GABAergic neurotransmission. In particular, it is clearly established that nerve lesion converts GABA into an excitatory neurotransmitter through BDNF-induced KCC2 downregulation and downstream Cl^- intracellular accumulation (Boulenguez et al 2010). Whether this mechanism also contributes to BDNF i.t.-induced hyperalgesia and/or allodynia has also to be addressed in future studies.

Finally, in our aim to further characterize BDNF-induced hyperalgesia, we compared the effects of different pharmacological treatments in BDNF i.t. rats vs CCI-SN rats. Pharmacological compounds used to treat neuropathic pain such as tapentadol, duloxetine and pregabalin as well as other drugs such as clonazepam or agomelatin were assessed in both models. Overall, our data showed that BDNF i.t. rats responded similarly as CCI-SN rats to these drugs. Under acute treatments conditions, tapentadol and pregabalin reduced transiently hyperalgesia whereas duloxetine and clonazepam were ineffective. Interestingly, agomelatine, a new antidepressant with an innovative mechanism of action, exerted an inhibitory effect on hyperalgeia in both models. Agomelatin is an agonist at melatonergic MT1 and MT2 receptors and an antagonist at $5HT_{2B}$ and $5HT_{2C}$ receptors (de Bodinat et al., 2010), and both properties might explain its anti-hyperalgesic action. Melatonin plays a role in pain modulation (Ambriz-Tutui et al., 2009) and $5HT_{2C}$ blockade can lead to the enhancement of noradrenergic

transmission (Millan et al., 2003) which has previously been shown to reduce hyperalgesia (Bohren et al., 2013). Further investigations are needed to identify which action(s) of agomelatin really underlie its capacity to alleviate BDNF i.t. as well as CCI-SN-induced neuropathic pain.

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Legends to figures

Figure 1 : **Time-course development of mechanical hyperalgesia and allodynia in CCI-SN rats and BDNF i.t. rats.**

Pressure threshold values to trigger vocalization in the Randall-Selitto test (mechanical hyperalgesia, A, B) or hindpaw withdrawal in the von Frey filaments test (mechanical allodynia, C, D) were determined at various times (abscissa) after sciatic nerve ligation in CCI-SN rats (A, C) or intrathecal injection of BDNF in BDNF i.t. rats (B, D). Parallel measurements were made in sham-operated rats (A,C) and in control rats injected intrathecally with saline (B,D). Each point is the mean ± S.E.M. of 6-7 independent determinations.

* P<0.05, ** P < 0.01, *** P < 0.001 compared to respective controls, two way ANOVA repeated measures, Bonferroni test.

Figure 2 : Respective characteristics of maximal hyperalgesia induced by CCI-SN versus BDNF i.t. in rats.

Pressure threshold values to trigger hindpaw withdrawal and vocalization in the Randall-Selitto test were determined 15 days after sciatic nerve ligation in CCI-SN rats (A) or 10 days after intrathecal injection of BDNF in BDNF i.t. rats (B). Parallel measurements were made at the same times in sham-operated rats (A) and control rats injected intrathecally with saline (B). Each point is the mean ± S.E.M. of 6-7 independent determinations.

* P<0.05, ** P < 0.01, *** P < 0.001 compared to respective controls, Student's t test.

Figure 3 : Anti-hyperalgesic effects of acute administration of pregabalin (A, B), tapentadol (C, D) or agomelatine (E, F) in CCI-SN (A, C, E) and BDNF i.t. (B, D, F) rats.

Acute administration of pregabalin (30 mg/kg i.p.), tapentadol (10 mg/kg i.p.), agomelatine (45 mg/kg i.p.) or their respective vehicle was performed (0 on abscissa) 15 days post-surgery in

CCI-SN rats or 10 days after intrathecal injection of BDNF (3.0 ng) in BDNF i.t. rats. Pressure threshold values to trigger vocalization in the Randall-Selitto test were determined at various times after treatment (D1 : 24 h post-treatment). Each point is the mean ± S.E.M. of n independent determinations. C on abscissa : Control (naive) rats (prior to surgery or i.t. BDNF).
* P < 0.05 compared to respective values in vehicle-treated rats, two way ANOVA repeated measures, Bonferroni test.

Figure 4 : Anti-hyperalgesic effects of cyclotraxin B in CCI-SN (A, C) and BDNF i.t. (B, D) rats.
Acute administration of cyclotraxin B (20 mg/kg i.p.) or saline was performed (0 on abscissa) 15 days after surgery in CCI-SN rats (A,C) or 10 days after intrathecal injection of BDNF (3.0 ng) in BDNF i.T. rats (B,D). Pressure threshold values to trigger vocalization in the Randall-Selitto test were determined at various times (abscissa), up to day 20 (A) or day 12 (B) post-treatment. Each point is the mean + S.E.M. of n independent determinations.
C on abscissa : Control (naive) rats (prior to surgery or i.t. BDNF).
* P < 0.05 compared to respective values in vehicle-treated rats, two way ANOVA repeated measures, Bonferroni test.
C,D : Bars are the mean + S.E.M. of AUCs (g x min) derived from the time-curves in CCI-SN (C from A) or BDNF i.t. (D from B).
* P < 0.05, *** P < 0.001 compared to respective controls, Student's t test.

Figure 5 : Spinal cord levels of transcripts encoding ATF3, OX42 and IL6 in CCI-SN (A) and BDNF i.t. (B) rats.
Real-time quantitative RT-PCR determinations were made in dorsal lumbar enlargement 15 days after CCI-SN (A, hemi-cord ipsilateral to CCI) or 24 h and 10 days after BDNF i.t. (B). Parallel measurements were made in respective controls. Data are expressed as the ratio of specific mRNA over GAPDH mRNA [R.Q.(A.U.)]. Each bar is the mean + S.E.M. of 6-12 independent determinations.
* P < 0.05, ** P < 0.01 compared to respective levels in sham-operated (CCI-SN) or saline-treated (BDNF i.t.) rats, Student's t test.

Figure 6 : Spinal cord levels of transcripts encoding BDNF and NR2B in CCI-SN (A) and BDNF i.t. (B) rats.

Real-time quantitative RT-PCR determinations were made in dorsal lumbar enlargement 15 days after CCI-SN (A, hemi-cord ipsilateral to CCI) or 24 h and 10 days after BDNF i.t. (B). Parallel measurements were made in respective controls. Data are expressed as the ratio of specific mRNA over GAPDH mRNA [R.Q.(A.U.)]. Each bar is the mean + S.E.M. of 6-12 independent determinations.

* $P < 0.05$, compared to levels in sham-operated (CCI-SN) or saline-treated (BDNF i.t.) rats, Student's t test.

Figure 7 : Immunohistochemical labeling of the microglial markers Iba1 and Phospho-P38 in the dorsal lumbar enlargement of the spinal cord in CCI-SN and BDNF i.t. rats.

A - Typical photomicrographs showing Iba1 and P-p38 immunoreactive cells (black dots) in the superficial layers (I,II) area within the dotted line of the dorsal horn of the lumbar enlargement of the spinal cord from a sham rat and a CCI-SN rat 15 days after surgery, and from a BDNF i.t. and a saline i.t. rat, 10 days after i.t. injection.

B – Bar graphs of the number (mean + S.E.M.) of immunolabeled cells per section in 15-30 sections from 3-4 rats per condition.

* $P<0.05$, *** $P<0.001$, one way ANOVA, Newman-Keuls test (CCI-SN) or Student's t test (BDNF i.t.).

Figure 1

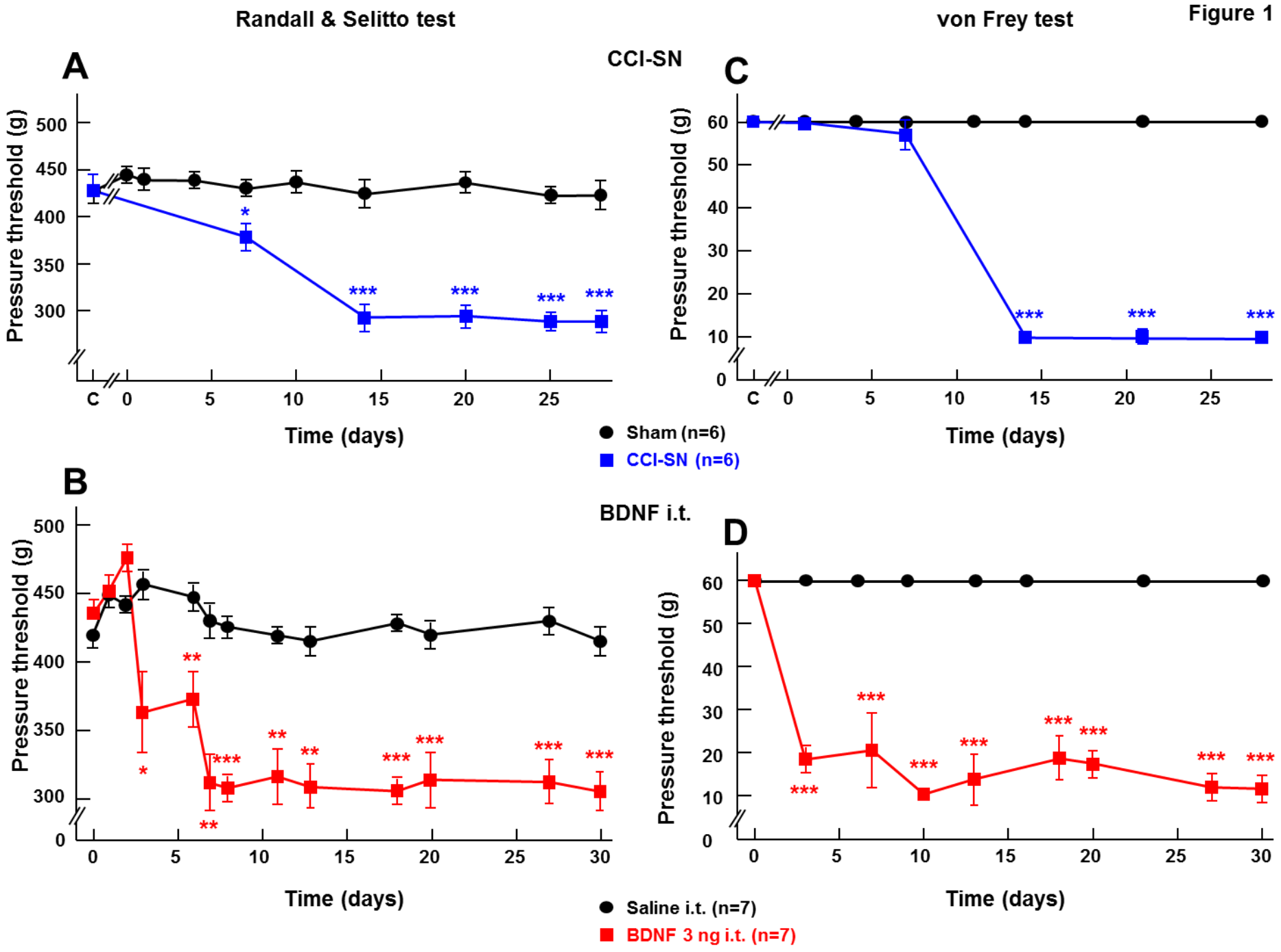

Randall & Selitto test
von Frey test
A
CCI-SN
C
Pressure threshold (g)
Time (days)
Sham (n=6)
CCI-SN (n=6)
B
BDNF i.t.
D
Saline i.t. (n=7)
BDNF 3 ng i.t. (n=7)

Figure 2

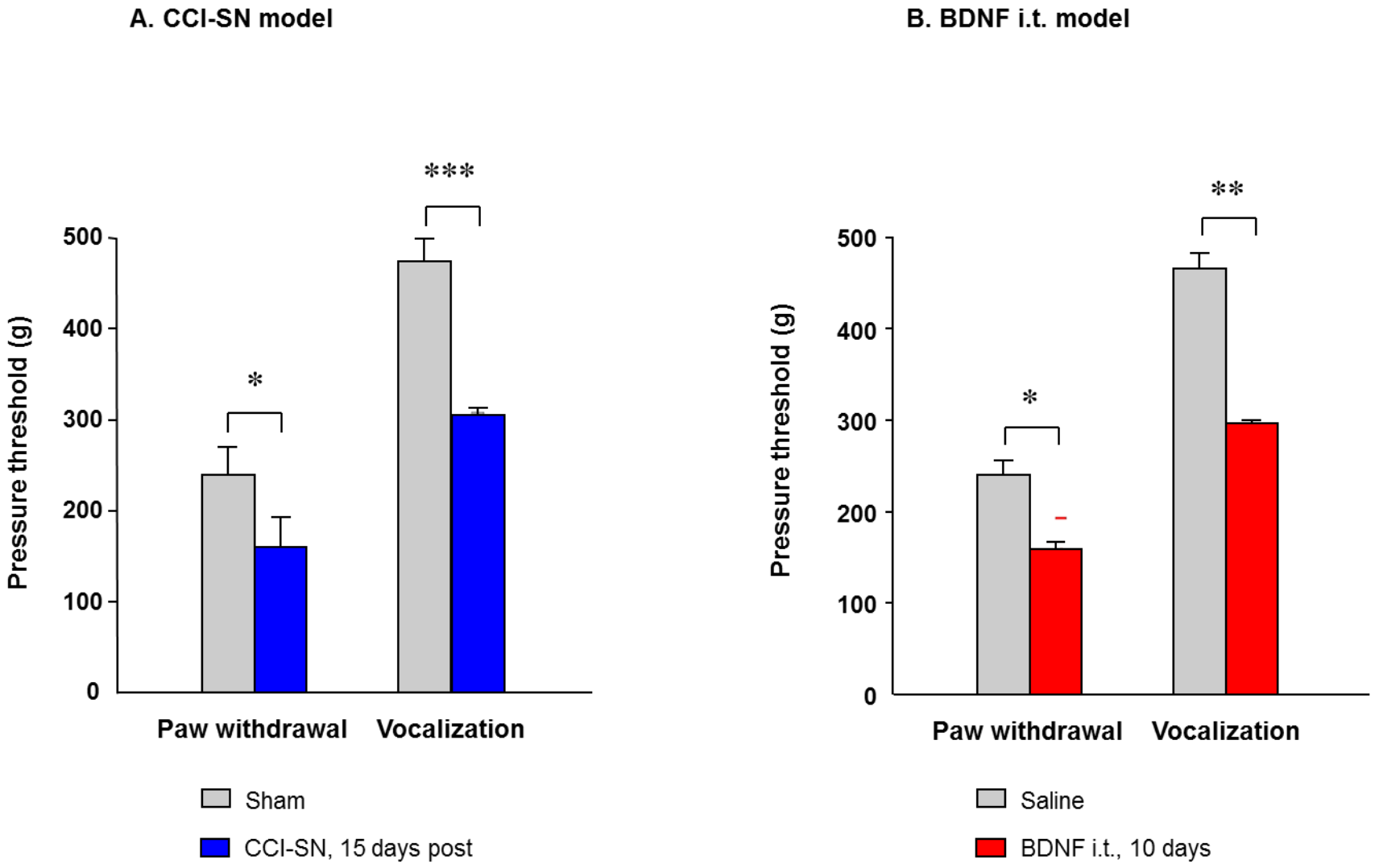

A. CCI-SN model
Pressure threshold (g)
500
400
300
200
100
0
*
***
Paw withdrawal
Vocalization
Sham
CCI-SN, 15 days post
B. BDNF i.t. model
Pressure threshold (g)
500
400
300
200
100
0
*
**
Paw withdrawal
Vocalization
Saline
BDNF i.t., 10 days

Figure 3

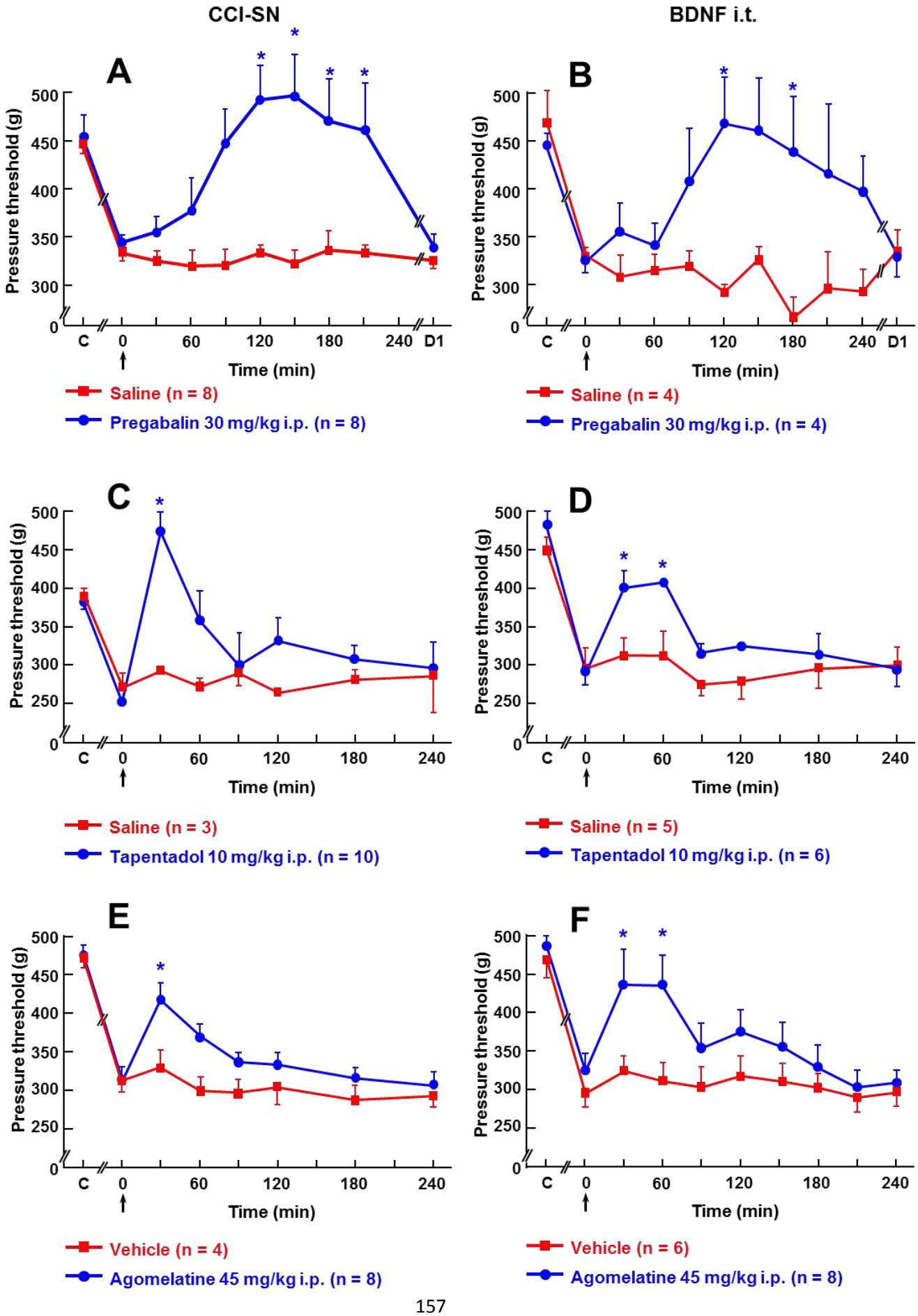

CCI-SN
BDNF i.t.
A
B
C
D
E
F
Pressure threshold (g)
Time (min)
C
0
60
120
180
240
D1
Saline (n = 8)
Pregabalin 30 mg/kg i.p. (n = 8)
Saline (n = 4)
Pregabalin 30 mg/kg i.p. (n = 4)
Saline (n = 3)
Tapentadol 10 mg/kg i.p. (n = 10)
Saline (n = 5)
Tapentadol 10 mg/kg i.p. (n = 6)
Vehicle (n = 4)
Agomelatine 45 mg/kg i.p. (n = 8)
Vehicle (n = 6)
Agomelatine 45 mg/kg i.p. (n = 8)

Figure 4

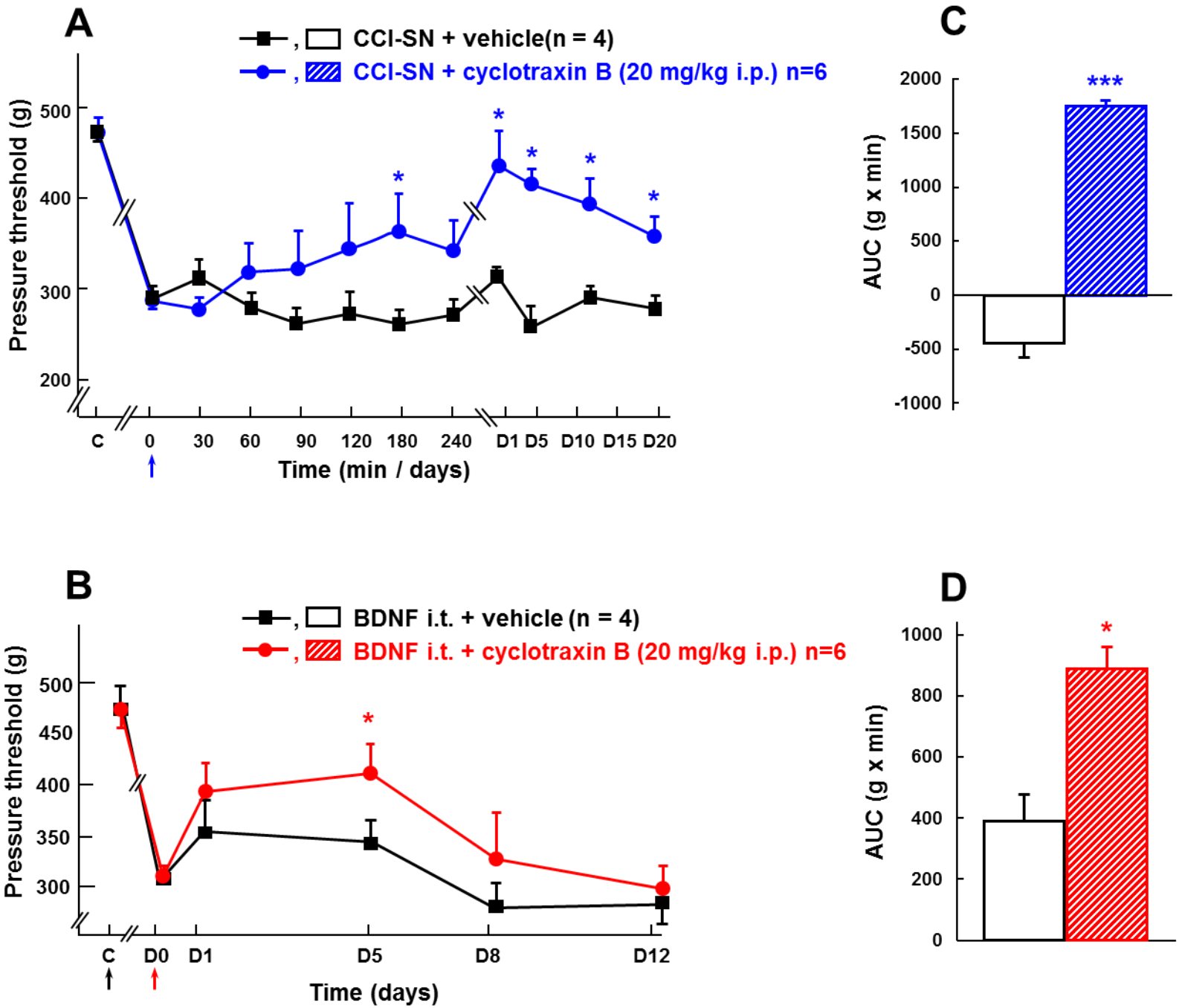

A
CCI-SN + vehicle(n = 4)
CCI-SN + cyclotraxin B (20 mg/kg i.p.) n=6
Pressure threshold (g)
500
400
300
200
C
0
30
60
90
120
180
240
D1
D5
D10
D15
D20
Time (min / days)
*
C
AUC (g x min)
2000
1500
1000
500
0
-500
-1000
***
B
BDNF i.t. + vehicle (n = 4)
BDNF i.t. + cyclotraxin B (20 mg/kg i.p.) n=6
Pressure threshold (g)
500
450
400
350
300
C
D0
D1
D5
D8
D12
Time (days)
*
D
AUC (g x min)
1000
800
600
400
200
0
*

Figure 5

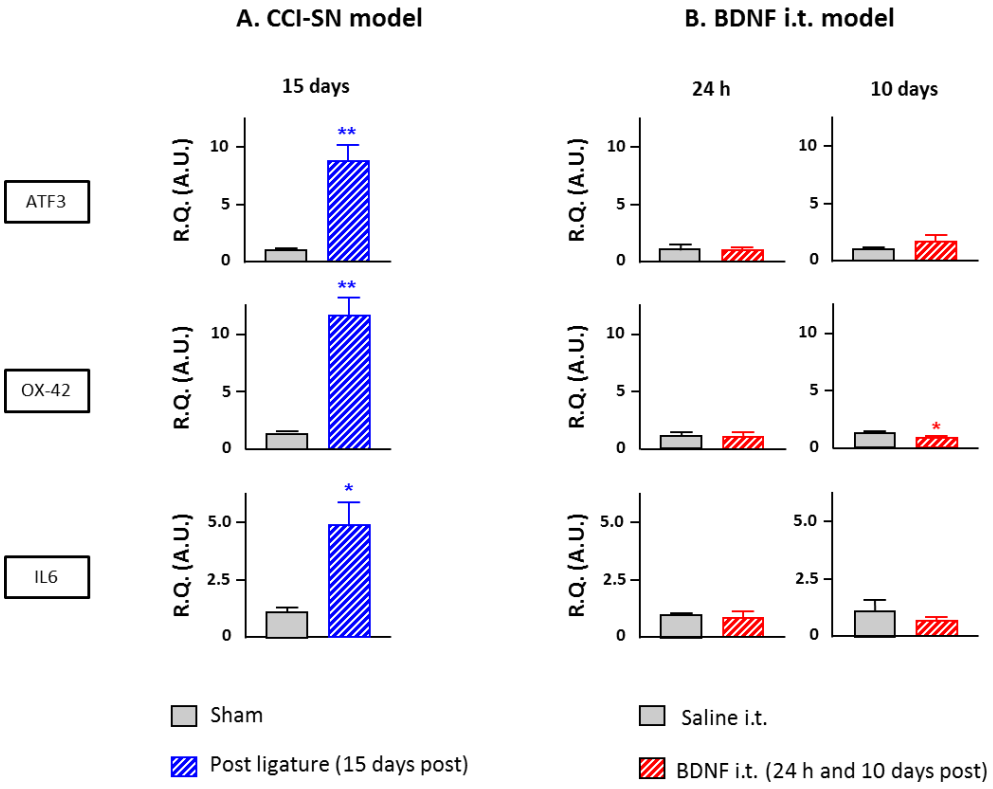

A. CCI-SN model
B. BDNF i.t. model
15 days
24 h
10 days
ATF3
OX-42
IL6
R.Q. (A.U.)
10
5
0
5.0
2.5
**
*
Sham
Post ligature (15 days post)
Saline i.t.
BDNF i.t. (24 h and 10 days post)

Figure 6

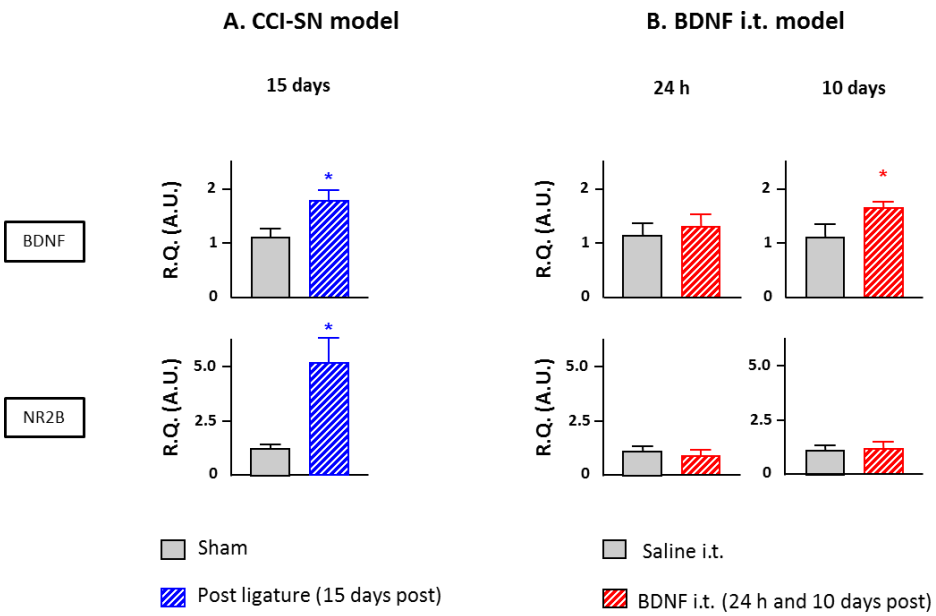
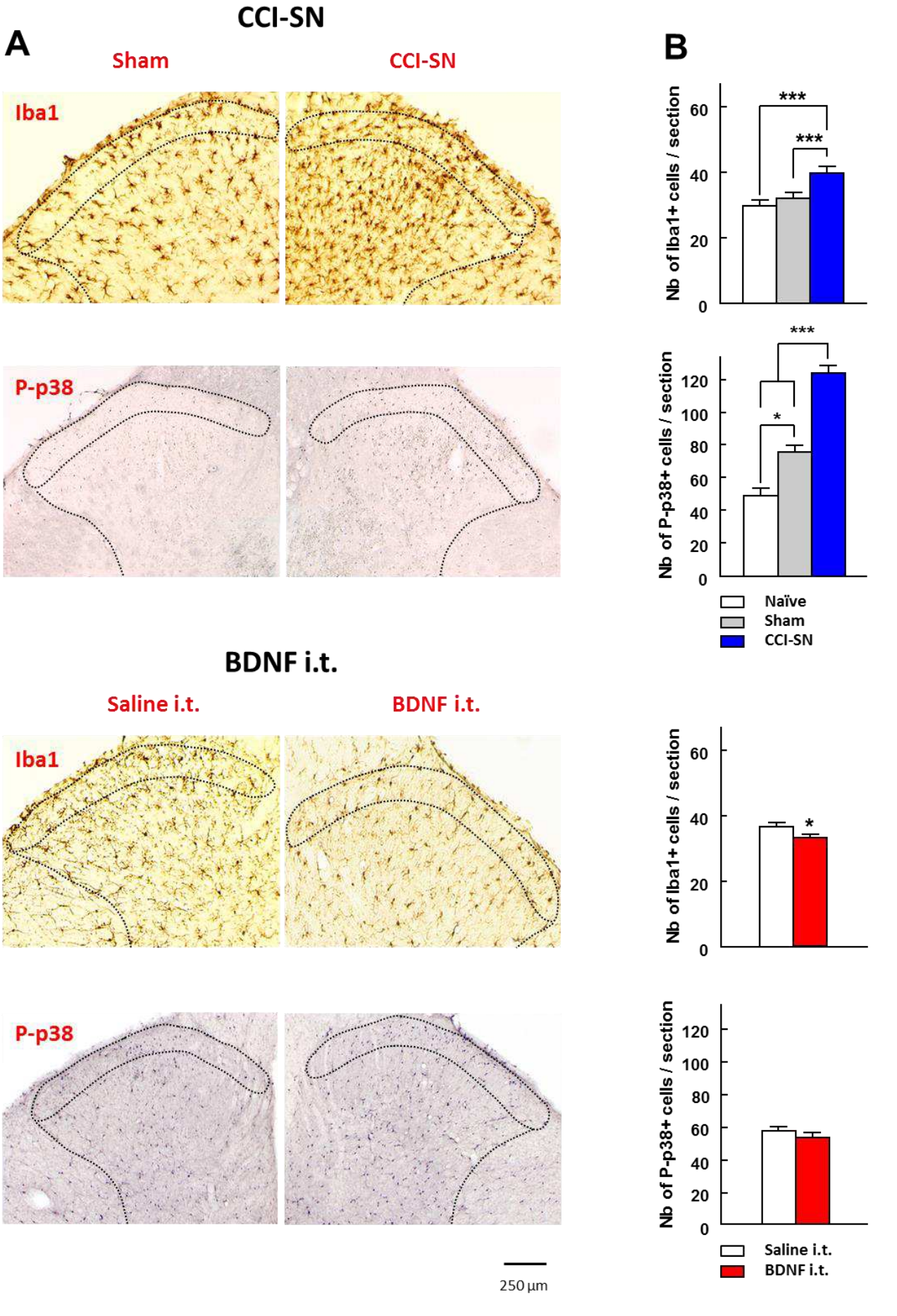

A. CCI-SN model
B. BDNF i.t. model
15 days
24 h
10 days
BDNF
NR2B
R.Q. (A.U.)
2
1
0
5.0
2.5
*
Sham
Post ligature (15 days post)
Saline i.t.
BDNF i.t. (24 h and 10 days post)

Figure 7


CCI-SN
A
Sham
CCI-SN
Iba1
P-p38
B
Nb of Iba1+ cells / section
Nb of P-p38+ cells / section
***
*
Naïve
Sham
CCI-SN
BDNF i.t.
Saline i.t.
BDNF i.t.
250 µm

DISCUSSION GENERALE

CHAPITRE I : RECHERCHE DE NOUVEAUX MODELES DE DOULEURS NEUROPATHIQUES

I.1. Définition d'un modèle

Un modèle animal est un compromis expérimental dans lequel un système expérimental simple est utilisé pour l'étude d'un système bien plus complexe et bien moins immédiatement accessible. Dans le cas d'une pathologie, l'élaboration d'un modèle vise à la transposition d'un syndrome de l'homme vers l'animal. L'utilisation d'un modèle permet d'étudier et de comprendre, sur de grands échantillons, les mécanismes impliqués dans cette pathologie et de tester différents traitements visant à en réduire les symptômes.
Le problème des modèles animaux, particulièrement dans le cas de modèles impliquant des tests comportementaux, est qu'ils peuvent être biaisés par une évaluation subjective de l'expérimentateur. Il est donc nécessaire de définir des critères clairs de validité d'un modèle. Ces critères ont été énoncés par McKinney et Bunney (1969) pour un modèle animal de dépression et précisés ensuite par Willner (1984). Tout d'abord, le modèle doit être homologue *(construct validity)*, c'est-à-dire qu'il doit y avoir une certaine correspondance entre les données étiologiques de la pathologie humaine et la procédure utilisée pour générer le modèle. Le modèle doit être isomorphe *(face validity),* et donc présenter des symptômes et une phénoménologie ressemblant le plus possible à ceux observés chez l'homme. Le modèle doit être prédictif *(predictive validity)* et donc répondre aux traitements pharmacologiques ou autres comme la pathologie humaine. Enfin le modèle doit être reproductible non seulement d'un expérimentateur à un autre mais aussi d'un groupe d'animaux à un autre, dans la même espèce/lignée.

I.2. Application à la douleur

Dans le cadre de la modélisation de la douleur neuropathique, les différents modèles qui sont utilisés répondent le plus souvent aux critères rappelés ci-dessus.
Ainsi, par exemple, le caractère « homologue » est validé dans le modèle de Bennett et Xie (1988) où la douleur neuropathique est générée par une lésion neurale (ligature du nerf sciatique). Par ailleurs, les phénomènes d'allodynie et d'hyperalgésie retrouvés dans ce même modèle sont isomorphes des symptômes douloureux qui caractérisent la pathologie humaine. Enfin, le caractère prédictif du modèle est validé par l'efficacité des agents pharmacologiques

utilisés chez l'homme à réduire les symptômes d'allodynie et d'hyperalgésie induits par la ligature du nerf sciatique chez l'animal.
Les deux modèles que nous avons étudiés (Transection complète de la moelle épinière et injection intrathécale de BDNF) ont certes donné lieu à quelques publications mais n'ont jamais été véritablement validés. Le but de ce travail de thèse a été de caractériser ces deux modèles non seulement d'un point de vue comportemental, mais aussi d'un point de vue pharmacologique et physiopathologique.

CHAPITRE II : LA TRANSECTION SPINALE ET L'INJECTION INTRATHECALE DE BDNF COMME NOUVEAUX MODELES VALIDES DE DOULEUR NEUROPATHIQUE

II.1. Le modèle de section complète de la moelle épinière (SCT)

Il existe de nombreux modèles de lésion de moelle épinière, les plus utilisés étant les modèles de contusion, compression ou hemi-section. Ces modèles sont utilisés parce qu'ils reproduisent assez bien le type de lésion observée chez l'homme. Cependant, ces procédures sont imparfaites et critiquables dès lors qu'elles nécessitent une laminectomie produisant une altération des méninges, une hémorragie ainsi que la perte de liquide céphalo-rachidien pouvant induire des perturbations non spécifiques de la lésion médullaire stricto sensu. De plus une grande variabilité interindividuelle existe dans un groupe d'animaux subissant ce type de lésion. En effet, l'étendue de la lésion est difficile à contrôler, les variations à ce niveau engendrent des conséquences différentes pour chacun des rats, et une grande hétérogénéité en termes de douleur (Basso et al., 1996; Crown et al., 2006). Il devient alors difficile d'interpréter les résultats notamment lorsqu'il s'agit d'évaluer l'efficacité d'agents pharmacologiques.
Le modèle de transection spinale que nous utilisons a été élaboré par Didier Orsal (Antri et al., 2003) pour l'étude de la locomotion. Les lésions complètes chez l'homme sont –heureusement- extrêmement rares. La classification d'un patient « ASIA-A », utilisée pour définir un patient ayant une lésion complète, s'applique en fait à toute personne ne ressentant soit plus aucune sensation au niveau sacré S4-S5, soit seulement quelques sensations localisées dans des zones dites de préservation partielle. Ces dernières témoignent en réalité d'une lésion incomplète chez des patients ASIA-A. La question de la pertinence des résultats obtenus chez l'animal SCT et de leur transposition chez l'homme se pose donc légitimement. Cependant, bien que le modèle de transection spinale ait le même inconvénient que ceux de compression, à savoir la nécessité

d'effectuer une laminectomie, celui-ci présente l'avantage d'être hautement reproductible (Onifer et al., 2007). De plus, ce modèle induit une allodynie spécifiquement localisée au niveau de la lésion ainsi qu'une hyper-réflexie au niveau des pattes postérieures permettant de tester l'efficacité d'agents pharmacologiques.

Un nouveau modèle de lésion de moelle épinière dénommé « air gun impactor » (Marcol et al., 2012) pourrait être particulièrement intéressant pour l'étude de douleurs neuropathiques d'origine centrale. Ce modèle consiste en l'application d'air à une pression de 220 kPa pendant 0,1 seconde au niveau de la moelle thoracique T9-T11. Les avantages de ce modèle sont (i) qu'il ne requiert qu'une faible intervention (trépanation légère) avant la lésion proprement dite de la moelle épinière, (ii) qu'il permet une très bonne reproductibilité de l'étendue de la lésion d'un animal à un autre et (iii) qu'il conduit à un profil de lésion observable en fMRI semblable à celui généralement observé après lésion de la moelle épinière chez l'homme.

II.2. Les modèles de lésion de nerf périphérique

Les modèles de douleur neuropathique d'origine périphérique les plus utilisés sont le modèle de transection de nerf (Wall *et al.*, 1979), et les différents modèles de ligature de nerf (Bennett & Xie, 1988). Le modèle de section de nerf étant assez controversé de par sa pertinence limitée au niveau clinique et l'autotomie qu'elle engendre chez les animaux (Riopelle, 1992), les modèles de ligature du nerf sciatique ou de ses branches se sont imposés comme reflétant le plus la pathologie chez l'homme. Ces modèles présentent l'avantage d'être relativement peu invasifs, rapides à mettre en œuvre, et ils reproduisent véritablement la compression d'un nerf qui peut survenir chez l'homme et les symptômes qui en découlent (allodynie, hyperalgésie). Cependant, de nombreux inconvénients inhérents à ces modèles les rendent imparfaits. Tout d'abord, la ligature se fait au niveau du nerf sciatique dont les trois branches parviennent aux ganglions des racines dorsales mais contiennent aussi des fibres motrices qui rejoignent des racines ventrales. Ceci peut compliquer l'interprétation des résultats obtenus dans les tests de nociception étant donné que ceux-ci se basent le plus souvent sur l'observation d'une réponse motrice après stimulation mécanique ou thermique. Ensuite, l'introduction d'un corps étranger, que ce soit des fils de soie ou du catgut peut, en elle-même, induire une inflammation (Maves et al., 1993) et contribuer à la douleur neuropathique de manière non spécifique à la compression du nerf. Il en est de même pour l'incision de la peau inhérente à l'intervention chirurgicale. La neuroinflammation, reflétée par l'activation, entre autres, de cytokines pro-inflammatoires et par l'activation microgliale, ne peut être seulement attribuée à la ligature en elle-même. Ainsi, la

résultante de ces ligatures au niveau des paramètres modifiés tant au plan cellulaire que moléculaire est-elle difficilement identifiable. Dès lors, il peut être hasardeux, voire erroné, de corréler directement les modifications cellulaires observables au niveau de la moelle épinière et des GRD avec la douleur neuropathique.
De plus, les animaux ne développent pas tous une neuropathie après ligature du nerf sciatique, rendant l'exploitation des expériences (financièrement mais aussi) éthiquement difficile. Cette variabilité dans le nombre de rats développant une allodynie/hyperalgésie provient très probablement de l'inconstance au serrage des fils de ligature.

II.3. Le modèle d'injection intrathécale de BDNF

Le modèle BDNF i.t. possède l'avantage de ne nécessiter aucune chirurgie, et n'entraine aucune inflammation. De plus, après injection intrathécale de BDNF (3 ng), on dispose d'un pourcentage d'animaux neuropathiques bien supérieur à celui après CCI-SN. En effet, 100% des animaux injectés avec le BDNF développent une allodynie (40% chez les CCI-SN) et 75% d'entre eux développent également une hyperalgésie (60% chez les CCI-SN). Cependant, comme pour le modèle CCI-SN, le BDNF affecte non seulement la corne dorsale mais aussi la corne ventrale de la moelle épinière, amenant le même problème que chez les animaux porteurs d'une ligature du nerf sciatique. En effet, le BDNF augmentant l'excitabilité des motoneurones (Gonzalez et Collins, 1997), les réactions comportementales observées chez l'animal BDNF i.t. pourraient être -au moins en partie- attribuées à cet effet. Il devient alors indispensable de valider le modèle en tant que modèle de douleur. Nous l'avons donc soumis à des agents pharmacologiques connus pour être efficaces chez l'homme et après ligature du nerf sciatique chez le rat, tels que la prégabaline et le tapentadol, ainsi qu'à un antidépresseur au mécanisme d'action innovant, l'agomélatine. Tous ces composés ont exercé quasi les mêmes effets chez le rat BDNF i.t. comparé au rat CCI-SN, validant ainsi l'intérêt pharmacologique du modèle BDNF i.t.

Le BDNF ayant comme récepteur non seulement TrkB, mais aussi le récepteur p75, il était pertinent de se demander via quelle cible ce facteur neurotrophique induit des symptômes caractéristiques des douleurs neuropathiques. Les expériences avec injection de cyclotraxine B « en préventif » et « en curatif » de l'injection de BDNF apportent un premier élément de réponse du fait que ce bloquant sélectif de TrkB prévient et diminue à long terme l'hyperalgésie induite par le BDNF. Cependant, ces effets préventifs et curatifs sont de courte durée. On peut donc envisager que le récepteur TrkB ne soit pas le seul en jeu dans le processus d'hyperalgésie

induit par le BDNF. Ceci paraissait vraisemblable dans la mesure où l'implication du récepteur p75 dans les processus de douleurs neuropathiques a déjà été démontrée (Obata et al., 2006a). Afin de répondre à cette question, nous avons étudié les effets d'un nouvel agoniste spécifique du récepteur TrkB [synthétisé par le Dr D. Rognan, CNRS, Strasbourg], le composé MIML4-11, chez le rat. En fait, l'injection intrathécale de MIML4-11 induit une hyperalgésie de longue durée et de même amplitude que le BDNF laissant à penser que l'activation du récepteur TrkB est suffisante pour induire une hyperalgésie (Fig. 19). Il serait intéressant de valider le modèle « MIML4-11 i.t. » pharmacologiquement et de comparer les mécanismes cellulaires et moléculaires d'induction et de maintien de douleur neuropathique à ceux du modèle BDNF i.t. afin d'identifier les cibles spécifiquement induites par le récepteur TrkB.

Au total, les deux modèles que nous avons étudiés semblent être de bons modèles dans la mesure où ils satisfont à au moins 2 des 3 critères rappelés plus haut.

Ainsi le modèle de transection de moelle épinière est pertinent dans la mesure où, bien qu'il ne soit pas totalement « homologue » car la section totale ne se produit que très rarement chez l'homme, on retrouve un « isomorphisme » au niveau des symptômes ainsi qu'une valence « prédictive » satisfaisante au regard de l'efficacité des traitements en clinique humaine. De plus, ce modèle est reproductible : 100% des animaux développent une allodynie après l'opération dans toutes les expériences qui ont été conduites.

Concernant le modèle BDNF i.t., il est impossible de parler d'homologie, l'injection intrathécale de BDNF étant sans rapport avec les évènements (traumatismes, traitements anticancéreux, intervention chirurgicale, …etc) à l'origine de douleurs neuropathiques chez l'homme. Cependant, ce modèle est particulièrement intéressant du fait qu'il satisfait au caractère d'« isomorphisme » en ce qui concerne les symptômes d'allodynie et d'hyperalgésie. De plus, les traitements anti-allodyniques/anti-hyperalgésiques efficaces chez l'homme et chez l'animal CCI-SN sont également efficaces dans ce modèle, montrant son intérêt «prédictif » en termes de pharmacologie. Sans compter qu'il s'agit d'un modèle bien plus facile à mettre en œuvre du fait de l'absence de chirurgie. Nos modèles étant validés, nous avons donc entrepris d'en approfondir l'étude à l'aide d'approches pharmacologiques, biochimiques et immunohistochimiques appropriées.

CHAPITRE III : EFFICACITE DES TRAITEMENTS PHARMACOLOGIQUES DANS LES DIFFERENTS MODELES ETUDIES

Les douleurs neuropathiques sont assez difficiles à traiter chez l'homme, les antalgiques classiques étant inefficaces et les traitements couramment utilisés n'ayant qu'une efficacité relative tout en induisant des effets indésirables (prise de poids, des nausées, des difficultés au plan sexuel, … etc cf Baastrup et al., 2008). Classiquement, les antidépresseurs ainsi que les anticonvulsivants ou leur combinaison sont les plus communémément utilisés. Les douleurs neuropathiques centrales sont, de manière générale, plus difficiles à traiter que les douleurs neuropathiques périphériques. On peut ainsi remarquer que pour les traitements pour lesquels il y a eu des essais cliniques contrôlés, leur efficacité était bien plus grande chez les patients souffrant de douleurs d'origine périphérique (Finnerup et al., 2010). Certains traitements utilisés pour soulager les douleurs neuropathiques *périphériques* se révèlent même inefficaces dans le cas de lésion de moelle épinière (par exemple l'application de patches de lidocaïne).
Cette différence se retrouve aussi dans les modèles expérimentaux de douleur que nous avons étudiés. Ainsi, la prégabaline et la gabapentine administrées de manière aiguë réduisent complètement, mais de manière transitoire, les phénomènes de douleur neuropathique dans les modèles de ligatures du nerf sciatique et d'injection i.t. de BDNF, tandis que ces mêmes traitements se sont révélés complètement inefficaces pour réduire la douleur neuropathique chez les rats médullo-lésés. De même, les composés opiacés tels que la morphine ou le tapentadol ont dû être administrés à des doses 2 à 3 fois plus fortes chez les animaux médullo-lésés que dans les autres modèles pour rétablir une sensibilité normale chez l'animal.

Afin d'évaluer le rôle de la sérotonine après lésion de la moelle épinière, nous avons recherché les effets éventuels de traitements avec le 8-OH-DPAT, un agoniste 5-$HT_{1A/7}$, l'ondansetron, un antagoniste 5-HT_3, et le naratriptan, un agoniste 5-$HT_{1B/1D}$. Le 8-OH-DPAT s'est révélé particulièrement efficace pour réduire les déficits locomoteurs et activer la marche automatique après section de la moelle épinière (Antri et al., 2003), ainsi que les douleurs neuropathiques d'origine périphérique (Sanchez et al., 1995; Pedersen et Blackburn-Munro, 2006). L'ondansetron permet de réduire les douleurs neuropathiques centrales suite à une contusion de moelle épinière tandis que le naratriptan s'est montré efficace pour diminuer les douleurs d'origine périphérique au niveau du territoire céphalique. Cependant, dans notre modèle SCT, le 8-OH-DPAT est resté sans effet sur les douleurs induites par la lésion de la moelle épinière, alors que le F13640, un agoniste sélectif des récepteurs 5-HT_{1A}, pouvait réduire l'allodynie mécanique après lésion photochimique de la moelle épinière (Colpaert et al., 2004).

On peut donc se demander si cette différence entre nos données et celles de Colpaert et al. (2004) d'action tient simplement à la différence de modèle utilisé ou à l'agoniste utilisé. En effet, le 8-OH-DPAT étant aussi un agoniste des récepteurs 5-HT_7, ceux-ci pourraient contrebalancer l'effet dû aux récepteurs 5-HT_{1A} étant donné que le blocage des récepteurs 5-HT_7 exerce des effets anti-allodyniques (Amaya-Castellanos et al., 2011) et anti-hyperalgésiques (Viguier et al., 2012). Dès lors, il serait intéressant de tester l'efficacité du F13640 dans le modèle dans les mêmes conditions. L'ondansetron non plus n'a pas réduit les douleurs neuropathiques dans notre modèle SCT, contrairement à ce qui a été rapporté dans un modèle de compression de la moelle épinière où l'allodynie au niveau de la lésion était réduite par l'administration de cet antagoniste des récepteurs 5-HT_3 (Chen et al., 2009). Mais, dans cette étude, l'injection d'ondansetron avait été faite de manière répétée montrant la nécessité d'un traitement chronique pour qu'il soit efficace.

Le baclofène, agoniste des récepteurs GABA (B), est généralement utilisé pour le traitement de la spasticité après lésion de la moelle épinière (Lewis et Mueller, 1993). Dans la mesure où l'injection intrathécale de baclofène réduit la douleur au niveau des pattes postérieures après compression de la moelle épinière (Hama et Sagen, 2012), nous nous sommes demandé quel pourrait être son effet dans notre modèle de section de moelle. L'injection de baclofène par voie systémique a réduit l'allodynie au niveau de la lésion, suggérant que cette manifestation comportementale de douleur neuropathique pouvait être sous-tendue, au moins en partie, par un déficit au niveau de la transmission GABAergique.

L'un des traitements pharmacologiques ayant diminué les douleurs neuropathiques d'origine centrale dans notre modèle SCT est l'administration aiguë de kétamine. Cet antagoniste des récepteurs NMDA est efficace pour réduire les douleurs neuropathiques chez l'homme (Kim et al., 2013). Ainsi, l'allodynie consécutive à la section de la moelle épinière pourrait résulter non seulement d'un déficit GABAergique mais aussi d'une activation de la « voie excitatrice NMDA ». Dans la mesure où la kétamine présente des effets indésirables à haute dose, tels que des hallucinations (Bredlau et al., 2013), et que le baclofène induit des phénomènes de sédation ou de somnolence (Dario et Tomei, 2004), leur utilisation sur le long terme pourrait être délétère chez les patients médullo-lésés. La combinaison de ces deux agents pharmacologiques, baclofène en injection intrathécale et kétamine par voie systémique, pourrait cependant être une solution à envisager étant donné qu'un effet synergique de cette combinaison sur la douleur neuropathique centrale a déjà été montré (Hama et Sagen, 2012).

Le clonazepam a souvent été recommandé pour le traitement des douleurs neuropathiques chez l'homme. Or, dans les trois modèles que nous avons étudiés, il n'y a eu aucune réduction de

l'allodynie ou de l'hyperalgésie. En réalité, récemment, des études contrôlées ont montré que cette benzodiazépine n'avait aucune efficacité anti-hyperalgique (Corrigan et al., 2012).

L'efficacité du tapentadol a été démontrée dans des modèles de douleur neuropathique d'origine périphérique (Michot et al., 2012) ainsi que chez l'homme souffrant de différents types de douleurs chroniques (Taylor et al., 2013). En accord avec ces données, le tapentadol a exercé une puissante action anti-hyperalgésique chez les rats BDNF i.t. De plus, le tapentadol s'est également révélé efficace pour réduire les douleurs neuropathiques d'origine centrale chez le rat ayant subi une transection médullaire. Ainsi, il pourrait être envisagé d'effectuer un essai clinique avec le tapentadol chez des patients médullo-lésés.

L'implication du BDNF dans les douleurs neuropathiques d'origine périphérique nous a amené à tester le potentiel d'un antagoniste du récepteur TrkB à diminuer les douleurs neuropathiques dans nos trois modèles. En accord avec l'augmentation d'expression du BDNF après ligature du nerf sciatique et l'induction d'allodynie et d'hyperalgésie par administration intrathécale de BDNF, la cyclotraxine B a réduit fortement l'hyperalgésie dans ces deux modèles. Cependant, elle ne réduit aucunement l'allodynie mécanique chez les rats médullo-lésés laissant à penser que la voie BDNF-TrkB n'est pas impliquée dans ce phénomène. D'ailleurs, au moment du traitement avec la cyclotraxine B, c'est-à-dire 30 jours après la section de la moelle épinière, nous n'avons pas observé d'augmentation de l'expression de BDNF, ni dans la moelle, ni dans les GRD. Cependant, comme une telle augmentation a été mise en évidence aux temps courts après la section spinale (aux jours 2 et 4 au niveau des GRD), il se pourrait que le BDNF soit impliqué davantage dans l'induction que dans le maintien des douleurs neuropathiques d'origine centrale. Pour tester cette hypothèse, il serait intéressant d'administrer la cyclotraxine B en préventif, juste avant la section spinale.

Malgré sa forte capacité à réduire l'hyperalgésie dans nos modèles de douleur neuropathique périphérique, la cyclotraxine B n'est probablement pas la molécule idéale pour un éventuel traitement anti-douleur chez l'homme. En effet, le récepteur TrkB est exprimé dans tout le système nerveux central, et son blocage conduirait inévitablement à de nombreux effets indésirables. Ainsi, il serait judicieux de chercher à savoir si le blocage du récepteur TrkB spécifiquement au niveau spinal, par injection intrathécale de la cyclotraxine B, peut produire les mêmes effets anti-hyperalgésiques que l'administration systémique de ce composé.

Etant donné leur utilisation courante chez l'homme, nous avons aussi testé différents antidépresseurs communément utilisés tels que l'amitriptyline, la duloxétine ainsi qu'un nouvel antidépresseur au mécanisme d'action innovant, l'agomélatine. Aussi bien chez le rat médullo-lésé, chez le rat porteur de ligatures sur le nerf sciatique que chez le rat injecté par voie

intrathécale avec le BDNF, l'amitriptyline et la duloxetine n'ont montré aucune capacité à réduire les comportements de type douloureux. En réalité, les données de la littérature ont clairement montré que ces antidépresseurs nécessitent une administration chronique pour exercer un effet à faible dose (Bomholt et al., 2005; Benbouzid et al., 2008a). Cependant, contrairement à l'amitriptyline et la duloxétine, l'agomélatine, à la fois agoniste des récepteurs mélatoninergiques MT1 et MT2 et antagoniste des récepteurs 5-HT_{2C} et 5-HT_{2B} de la sérotonine, administrée en aigu, a induit une diminution de la douleur neuropathique après ligature du nerf sciatique ou injection intrathécale de BDNF. Compte tenu des deux cibles pharmacologiques de l'agomélatine (cf ci-dessus), on peut se demander par quel mécanisme l'agomélatine a réduit les douleurs neuropathiques chez le rat. En réalité, des données de la littérature montrent qu'aussi bien l'activation des récepteurs de la mélatonine (Ambriz-Tututi et Granados-Soto, 2007 ; Ambriz-Tututi et al., 2009; Srinivasan et al., 2012) que l'augmentation de la libération de la noradrénaline induite par le blocage des récepteurs 5-HT_{2C} (Millan et al., 2003) peuvent exercer un effet antalgique. Des études complémentaires sont à envisager dans le but de savoir si seulement l'une de ses actions ou bien les deux sous-tendent les effets anti-hyperalgésiques de cet antidépresseur atypique.

L'un des résultats les plus étonnants concernant le modèle de section de la moelle épinière est l'inefficacité de la prégabaline alors que cet agent pharmacologique a un effet anti-hyperalgésique dans les deux autres modèles que nous avons étudiés ainsi que chez les patients médullo-lésés (Tzellos et al., 2008). L'inefficacité de la prégabaline, comme d'ailleurs celle de l'amitriptyline et de la duloxétine, dans notre modèle de douleur neuropathique par section spinale s'explique sans doute par le fait que nous avons administré ces composés de manière aiguë alors que chez l'homme ainsi que dans d'autres modèles de douleur expérimentale leur efficacité ne semble véritablement apparaître qu'au bout de plusieurs semaines de traitement. Cependant certaines études ont montré que la prégabaline en aigu, à la même dose que celle que nous avons utilisée, pouvait diminuer les réactions nocifensives chez le rat. Ainsi, chez des rats ayant subi une contusion de la moelle épinière, Baastrup et al. (2011) ont montré qu'une seule injection de prégabaline permettait de réduire l'allodynie au niveau de la lésion. La différence entre cette donnée et notre observation pourrait être liée aux procédures utilisées pour évaluer les comportements douloureux dans l'étude de Baastrup et al (2011) et dans la nôtre. De fait, les comportements que nous avons quantifiés (ébrouement, morsure, fuite) seraient plutôt des comportements médiés par la moelle (ébrouement) et le tronc cérébral (morsure, fuite) tandis que le test de « place/escape/avoidance » utilisé par Baastrup et al. (2011) impliquerait plutôt une composante affective et émotionnelle de la douleur, et donc davantage le cortex cérébral,

notamment l'aire cingulaire antérieure (LaGraize et al., 2004). Ainsi, la prégabaline pourrait avoir un impact surtout sur la composante émotionnelle de la douleur chez les animaux ayant subi une lésion de la moelle épinière. Il pourrait être intéressant d'évaluer, à l'aide du test « place/escape avoidance » (LaBuda & Fuchs, 2000), non seulement la prégabaline mais aussi les antidépresseurs (amitriptyline, duloxétine) qui sont restés inefficaces dans nos conditions expérimentales.

L'administration d'un inhibiteur de l'activation microgliale permettant de réduire les douleurs neuropathiques de type périphérique (Latremolière et al., 2008), il aurait été intéressant d'évaluer le potentiel d'agents pharmacologiques tels que la minocycline chez le rat médullo-lésé, d'autant que nous avons observé une forte activation microgliale après section de la moelle épinière. De même, le fluorocitrate, qui inhibe l'activation astrocytaire, serait à tester chez ces animaux étant donné la forte augmentation de GFAP après section de la moelle.

Les divers composés que nous avons testés dans nos modèles ont donc montré une efficacité différente selon que la lésion était centrale ou périphérique. Ces observations nous ont amené à nous demander quelle était l'origine de cette différence de réponse pharmacologique. Dans le but de répondre à cette question, nous avons entrepris d'analyser certaines des altérations cellulaires et moléculaires associées aux douleurs neuropathiques chez le rat médullo-lésé SCT et le rat injecté en intrathécal avec le BDNF comparés au rat CCI-SN porteur de ligatures sur le nerf sciatique.

CHAPITRE IV : MECANISMES PHYSIOPATHOLOGIQUES SOUS-TENDANT L'ALLODYNIE ET/L'HYPERALGESIE DANS LES MODELES SCT ET BDNF I.T.

IV.1. Modèle SCT

IV.1.a. Spasticité et hyper-réflexie

La section de moelle épinière induit une allodynie dans le territoire cutané au niveau de la lésion et une hyper-réflexie au niveau des pattes postérieures. L'hyper-réflexie, mesurée par le test des filaments de von Frey, se développe sur une quinzaine de jours après la section de moelle épinière, de même que la survenue d'une spasticité que nous avons pu observer mais que nous n'avons pas quantifiée. La spasticité et l'hyper-réflexie reflètent des modifications des propriétés des motoneurones spinaux. En effet, après section spinale, il a été rapporté une augmentation de

l'expression de l'isoforme constitutivement active du récepteur 5-HT_{2C} entrainant ainsi une augmentation des courants calciques entrants et donc une hyperexcitabilité des motoneurones spinaux (Murray et al., 2010). Par ailleurs, les phénomènes de plasticité et d'hyper-réflexie ont été corrélés à la diminution du transporteur KCC2, celle-ci entrainant aussi une hyperexcitabilité neuronale au niveau de la moelle lombaire. Bien que ces phénomènes de spasticité et d'hyper-refléxie soient associés à des douleurs chez l'homme (Werhagen et al., 2004), ce ne peut être le cas dans notre étude puisque le message nociceptif ne peut atteindre le cerveau chez le rat dont la moelle épinière a été complètement sectionnée au niveau thoracique (T8-T9).

IV.1.b. Allodynie

IV.1.b.1. Rôle de l'hyperexcitabilité neuronale

Les mécanismes cellulaires et moléculaires qui sous-tendent l'allodynie au niveau de la section spinale sont multiples et siègent à différents étages du nevraxe. Tout d'abord, il a été montré qu'au niveau spinal immédiatement rostral à la lésion, il y a une augmentation de l'activité neuronale qui est corrélée à l'apparition de l'allodynie (Scheifer et al., 2002; Hoheisel et al., 2003). Cette activation électrophysiologique semble être en relation, tant au niveau de la moelle qu'à celui des neurones de la voie spino-thalamique, avec une augmentation de l'activité des canaux sodiques (Zhang et al., 2005) et une expression accrue de la CaMKII, une kinase spécifiquement neuronale dont l'intervention se situe en amont de l'activation de ERK et de P38 dans les voies de signalisation intracellulaire. De fait, l'inactivation de CaMKII par injection intrathécale de l'inhibiteur KN-93 permet de réduire à la fois l'allodynie au niveau de la lésion et l'activité des neurones spinaux chez le rat ayant subi une contusion de la moelle épinière.

IV.1.b.2. Rôle de l'activation gliale

L'activation microgliale a un rôle important dans les phénomènes de douleur neuropathique. Celle-ci se fait principalement via les récepteurs purinergiques P2X et les récepteurs TLR. Dans notre modèle de section de la moelle épinière, nous avons pu montrer qu'il y a une augmentation de l'expression des gènes codant pour les récepteurs P2X4, P2X7 et TLR4. Ces récepteurs sont principalement exprimés au niveau des cellules microgliales et participent à l'inflammation via l'activation de la libération de cytokines. L'activation microgliale et astrocytaire, détectée par les marqueurs Iba1 et CD11b (OX-42) d'une part et GFAP d'autre part, est souvent corrélée aux douleurs neuropathiques. Elle est particulièrement nette dans le modèle SCT.

Les cytokines, bien qu'ayant un rôle important à jouer, sont plutôt des marqueurs de la neuroinflammation. L'implication des cytokines pro-inflammatoires dans la douleur neuropathique a été clairement démontrée que ce soit après la lésion d'un nerf périphérique ou celle de la moelle épinière (Latrémolière et al., 2008; Guptarak et al., 2013; Zhang et al., 2013).

Les cytokines permettent l'induction et le maintien des douleurs neuropathiques notamment par leur capacité à induire la LTP en augmentant l'expression de canaux NMDA et sodiques, mais aussi de par leur capacité à diminuer la transmission GABAergique (Ji et Suter, 2007; Gwak et al., 2008). En plus de l'activation des cytokines, on peut aussi noter l'importance de l'activation des chemokines dans les phénomènes d'induction de la douleur neuropathique. Les chemokines se caractérisent par leur rôle dans le recrutement des cellules immunitaires au niveau du site de la lésion. Ainsi, on peut observer, très tot après une lésion de nerf périphérique, une forte augmentation des taux tissulaires de chemokines, notamment MCP1 (CCL2) et de son récepteur CCR2 ainsi que de la fractalkine CX3CR1. Leurs rôles respectifs dans l'induction et/ou le maintien des douleurs neuropathiques d'origine périphérique ont été clairement démontrés (Dauvergne et al., 2013). Après une lésion de moelle épinière, il a également été rapporté une production accrue des chemokines MCP1 et CXCL12, corrélée à une augmentation du nombre de cellules immunitaires au niveau même de la lésion. L'injection de la chemokine CXCL12 à ce niveau provoque une augmentation locale du nombre de cellules souches précurseurs et de cellules immunitaires, et stimule la croissance axonale, amenant ainsi un espoir pour la réparation des troubles locomoteurs liés à une lésion médullaire. Cependant, il convient de rappeler que la croissance axonale non contrôlée est un des mécanismes de plasticité pouvant conduire aux douleurs neuropathiques (Macias et al., 2006).

Ainsi, il semble pertinent de penser que les cytokines, via l'activation microgliale, sont nécessaires à la mise en place de la douleur neuropathique.

Cette activation microgliale est reflétée par l'activation des MAPK, en particulier p38. D'ailleurs, dans le modèle de lésion médullaire par contusion, l'inhibition de l'activation microgliale par la minocycline entraine une diminution de l'activation de la MAPK p38 (Hains et Waxman, 2006). En réalité, dans ce même modèle, p38 est augmentée non seulement au niveau de la microglie mais aussi au niveau des astrocytes et des neurones dans la corne dorsale de la moelle épinière, et l'injection spinale d'un inhibiteur spécifique de p38 diminue l'allodynie au niveau de la lésion (Crown et al., 2008).

Les rôles respectifs des différentes cytokines et de l'activation microgliale dans la mise en place et le maintien des phénomènes douloureux dans le modèle SCT versus le modèle CCI-SN semblent distincts - au moins en partie - en accord avec l'idée que des mécanismes cellulaires et

moléculaires spécifiques sous-tendent les douleurs neuropathiques centrales et périphériques. En effet, la section de la moelle épinière semble induire - en général - une activation des astrocytes et de la microglie plus forte et plus soutenue dans le temps que la ligature du nerf sciatique. Par ailleurs, l'induction des cytokines pro-inflammatoires est également plus forte chez les rats SCT par rapport aux rats CCI-SN au 2ème jour après la chirurgie. Mais trois semaines plus tard, l'expression des cytokines IL6 et IL-1β est toujours augmentée au niveau de la moelle épinière et des DRG chez le rat CCI-SN tandis qu'on n'observe qu'une faible induction de ces cytokines chez le rat SCT. De même, l'expression du BDNF est augmentée plus longtemps chez le rat CCI-SN que chez le rat SCT (Fig 21). L'importance de l'inflammation et de l'activation microgliale dans le maintien de la douleur neuropathique pourrait être remise en cause notamment du fait que chez les animaux BDNF i.t., une hyperalgésie et une allodynie se développent en l'absence de toute activation gliale.

IV.1.b.3. Rôle de la plasticité medullaire

Parmi les mécanismes en cause dans la douleur neuropathique, la neuroplasticité revêt une place particulièrement importante du fait qu'elle est étroitement liée aux processus douloureux chroniques. Différents mécanismes de neuroplasticité peuvent être impliqués. Ainsi, la pousse neuritique anarchique (le « sprouting ») de fibres CGRP via l'activation de l'expression de GAP-43 dans la moelle épinière pourrait y contribuer (Christensen & Hulsebosch, 1997). Aujourd'hui, l'un des axes principaux des recherches sur les lésions médullaires concerne le développement de stratégies visant à améliorer, voire restaurer, la locomotion. Ces stratégies consistent principalement à promouvoir la repousse neuritique au niveau de la moelle épinière afin de permettre la reconnexion entre les neurones situés au-dessus et en-dessous de la lésion. Or, ces stratégies de repousse neuritique telles que l'implantation de cellules souches neurales adultes au niveau de la lésion ont certes amélioré les capacités locomotrices dans des modèles animaux appropriés mais ont aussi augmenté les douleurs neuropathiques (Hofstetter et al., 2005 ; Deumens et al., 2008). Il en est de même pour la transplantation d'astrocytes dérivés des GRP (« glial restricted precursors »). Ainsi, des astrocytes non différenciés ou différenciés par le Ciliary Neurotrophic Factor (CNTF) induisent une allodynie mecanique et une hyperalgésie thermique au niveau des pattes chez des rats ayant subi une contusion de la moelle épinière (Davies et al., 2008). Une induction de douleur neuropathique est aussi observée avec la transplantation de cellules de type « olfactory ensheathing cell » (OEC) qui sont très souvent utilisées pour stimuler les processus de régénération au niveau spinal (Mackay-Sim et al., 2008).

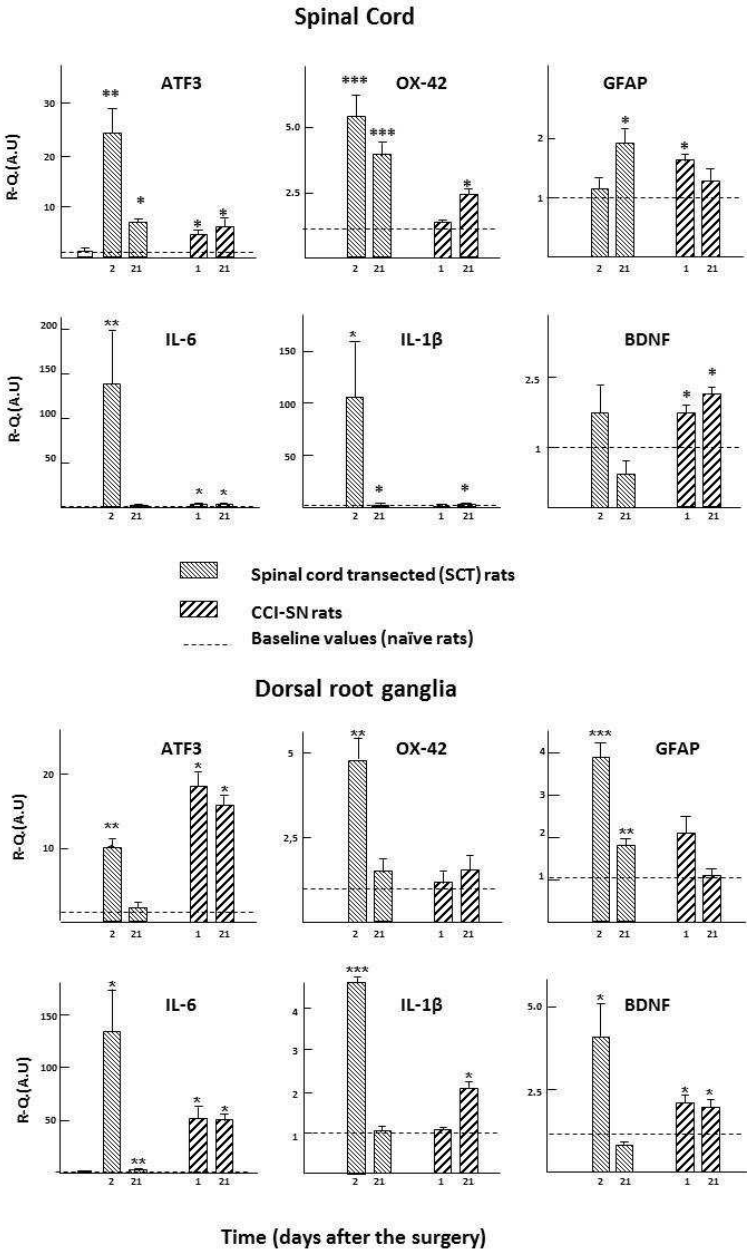


Figure 21:

Levels of mRNA encoding ATF3, OX-42, GFAP, IL-6, IL1-β and BDNF in the spinal cord and dorsal root ganglia at early (1 or 2 days) and late (21 days) time after surgery in SCT and CCI-SN rats. Bars represent the fold induction ± S.E.M. compared to respective control (n=5-12 rats for each determination).

** P<0.05, ** P<0.01, *** P<0.001 compared to respective values in « sham » rats*

Two ways ANOVA followed by Bonferroni test.

Dans ce cas, l'hyperalgésie pourrait être médiée par le BDNF (Lang et al., 2013). De fait, de par son activité trophique, le BDNF lui même est également utilisé pour promouvoir la repousse neuritique. Ainsi, l'injection de vecteurs viraux recombinants produisant du BDNF conduit certes à une récupération partielle de la locomotion chez le rat médullo-lésé mais aussi à une sensibilisation à des stimuli thermiques (chauds) nociceptifs et à une spasticité (Boyce et al., 2012). En accord avec ces observations, il a été montré récemment que l'activation du récepteur TrkB T1 du BDNF était impliquée dans les phénomènes d'allodynie chez le rat médullo-lésé (Wu et al., 2013). En plus de promouvoir la pousse neuritique, le BDNF joue aussi un role majeur dans l'induction de mécanismes pro-algésiques au niveau cellulaire. Ceci amène à un dilemme assez complexe où il ne pourrait n'y avoir la place pour le recouvrement que de la locomotion **ou** de la douleur. De plus, cette question souligne aussi l'importance de la barrière astrogliale qui, au final, empêche l'établissement de ces connexions aberrantes ; il est donc important de la conserver.

IV.2. Modèle BDNF i.t.

IV.2.a. Rôle de l'hyperexcitabilité neuronale

Plusieurs hypothèses peuvent être avancées quant aux mécanismes conduisant à une hyperalgésie et une allodynie chez les rats BDNF i.t. Tout d'abord, le BDNF peut sensibiliser les neurones de la moelle épinière via une augmentation de l'expression et de l'activation des récepteurs NMDA. D'ailleurs, l'administration d'un bloquant non spécifique des récepteurs NMDA réduit l'hyperalgésie thermique induite par une injection intrathécale de BDNF (Groth & Aanonsen, 2002). De fait, à court terme, la tyrosine kinase fyn se lie au récepteur TrkB lorsque celui-ci est phosphorylé, ce qui conduit à la phosphorylation activatrice de la sous unité NR2B du récepteur NDMDA. La piste de cette activation du récepteur NMDA comme responsable de l'hyperalgésie mécanique chez le rat BDNF i.t. mériterait d'être analysée, notamment avec des antagonistes NMDA tels que l'AP5 et des bloquants spécifiques de la sous unité NR2B tels que l'ifenprodil. Le BDNF semble aussi augmenter l'expression et l'activation de sous unités du récepteur AMPA de manière ERK- et NMDA- dépendante. Une expression accrue du récepteur AMPA sous l'action du BDNF a notamment été rapportée au niveau du noyau accumbens et de l'hippocampe (Wu et al., 2004; Li & Keifer, 2009; Li & Wolf, 2011). Par ailleurs, l'activation du récepteur AMPA, liée à la phosphorylation du résidu Ser831 de la sous unité GluR1, semble impliquée dans les phénomènes de douleurs neuropathiques et inflammatoires de manière directement

dépendante de la phosphorylation de la sous-unité NR2B (Lu et al., 2008; Katano et al., 2011). Le BDNF peut aussi moduler l'activité de différents canaux ioniques. Ainsi, il a été montré que, via son récepteur TrkB, le BDNF pouvait induire une dépolarisation membranaire au travers d'une activation du canal sodique tetrodotoxine (TTX) résistant Nav 1.9 (Blum et al., 2002). De plus, le rôle du BDNF dans la diminution de l'expression des canaux potassiques voltage-dépendants et des canaux « Ca^{2+} -activated K^{+} » (BK) au niveau des ganglions des racines dorsales a été montré dans des modèles de section du nerf sciatique et de neuropathie diabétique (Park et al., 2003; Cao et al., 2010, 2012). Comme ces modifications ralentissent le retour au potentiel membranaire de repos, il en résulte un accroissement de l'excitabilité neuronale sous l'action du BDNF.

En plus de son impact sur les canaux voltage-dépendants, le BDNF, également via son récepteur TrkB, peut diminuer l'expression du co-transporteur KCC2 (Rivera et al., 2002 ; Miletic & Miletic, 2008) et ainsi diminuer l'action inhibitrice du GABA, voire la transformer en une action excitatrice. Cependant nos expériences de qRT-PCR n'ont pas révélé de changements des taux tissulaires de l'ARNm de KCC2 dans nos conditions après injection intrathécale de BDNF. Quoi qu'il en soit, le rôle de KCC2 n'est pas à exclure étant donné que le BDNF exerce aussi des effets au niveau post-traductionnel pouvant entrer en jeu dans les modifications des courants « potassique » et « chlorure » (Boulenguez et al., 2010).

D'autre acteurs cellulaires que les canaux ou transporteurs ioniques mériteraient aussi d'être explorés comme par exemple les récepteurs TRP et les canaux ASIC. En effet, il a été montré que l'application de BDNF sur des neurones de ganglion de racine dorsale en culture augmentait leur expression du récepteur TRPV1 (Ciobanu et al., 2009). Enfin, il a été rapporté récemment que l'hyperalgésie mécanique induite par l'injection intrathécale de BDNF était au moins en partie médiée par l'activation du récepteur ASIC1A (Duan et al., 2012).

Dans notre modèle BDNF i.t, l'expression de fosb/Δfosb a été quantifiée par immunohistochimie. L'étude de ce marqueur a été motivée du fait de son implication dans les phénomènes d'activation persistante, notamment dans les phénomènes d'adaptations cérébrales et spinales en rapport avec le stress et les douleurs neuropathiques et inflammatoires (McClung et Nestler 2003 ; Luis-Delgado et al., 2006 ; Aouad et al., 2013). De plus, il semble que Δfosb puisse être co-exprimé avec le BDNF comme l'ont rapporté Nikulina et al. (2012). Dans notre modèle BDNF i.t., fosb est significativement augmenté par rapport aux animaux « véhicule », démontrant qu'au niveau de la moelle épinière, le BDNF induit des changements à long terme, qui pourraient être à l'origine de l'hyperalgésie induite par ce facteur neurotrophique. En particulier, une hyperexcitabilité neuronale pourrait constituer l'un de ces changements étant

donné que l'un des gènes cibles de fosb est la sous unité NR1 du récepteur NMDA (McClung et Nestler, 2003).

IV.2.b. Rôle de l'activation gliale

Nous avons noté une absence d'activation microgliale chez le rat BDNF i.t. En réalité, ceci peut s'expliquer par le fait que, dans les voies de signalisation impliquées dans la douleur neuropathique, le BDNF se situe plutôt en aval de l'activation microgliale et astrocytaire (Trang et al., 2009; Zhang et al., 2012). Néanmoins, dans notre étude, il convenait tout de même de vérifier qu'il n'y avait pas d'activation microgliale étant donné que la microglie, de même que les astrocytes, possèdent des récepteurs TrkB (Zhang et al., 2012).
Une douleur neuropathique n'impliquant pas d'activation microgliale est également observée chez les animaux allodyniques suite à une injection d'oxaliplatine. Ainsi, Zheng et al. (2011) rapportent que l'administration d'oxaliplatine ne provoque pas d'activation microgliale aussi bien dans la moelle épinière que dans le noyau spinal caudal du trijumeau (Sp5c), soulignant à nouveau l'existence de différences majeures entre la neuropathie provoquée par ce traitement et celle résultant de la ligature d'un nerf. Néanmoins, on ne peut exclure qu'il y ait une activation microgliale transitoire dans les premières heures suivant l'injection intrathécale de BDNF, notamment dans la phase d'induction de l'allodynie/hyperalgésie. Ce point mériterait d'être vérifié dans le cadre de perspectives de recherche au-delà du travail rapporté dans ce mémoire.

IV.2.b Rôle de la plasticité médullaire

Toutes ces modifications induites par le BDNF concourent à l'induction d'une LTP qui se maintient dans le temps du fait que le récepteur TrkB stimule la formation de nouvelles synapses et la pousse de boutons dendritiques en augmentant notamment l'expression de Rac1 et de GAP 43 (Jain et al., 2011 ; Lai et al., 2012). De plus, le BDNF augmente l'expression et la phosphorylation de PKMζ, une kinase impliquée dans l'induction et le maintien de la LTP (Melemedjian et al., 2013).
Tous ces phénomènes de neuroplasticité pourraient être maintenus dans le temps par un phénomène d'autoinduction du BDNF qui a pu être révélée par une augmentation de l'expression de son ARNm dans notre modèle BDNF i.t. dix jours après l'injection intrathécale du facteur neurotrophique. Ce phénomène d'auto-induction a déjà été documenté dans d'autres articles notamment *in vitro* (Yasuda et al., 2007) et *in vivo* au niveau du gyrus denté de l'hippocampe

(Wibrand et al., 2006). *In vitro*, cette auto-induction semble nécessiter l'activation de la MAPK ERK mais aussi celle des récepteurs NMDA. L'expression du gène codant le BDNF au niveau des GRD, et plus particulièrement celle des exons I et IV qui semble liée à l'activation neuronale via les kinases PKC et p38 et les facteurs de transcription CREB et NFκB (Morioka et al., 2013), mériterait d'être analysée de façon plus approfondie. Les nombreux articles évoquant la microglie comme type cellulaire permettant la libération de BDNF laissent envisager que celui-ci aurait une origine microgliale chez le rat BDNF i.t. (Trang et al., 2009). Cependant, l'absence d'activation microgliale après l'injection i.t. de BDNF rend l'hypothèse de la libération du BDNF par la microglie peu vraisemblable. En réalité, le BDNF peut aussi être produit et libéré par les neurones des GRD (Pezet et al., 2002b) et par les astrocytes (Zhang et al., 2012). Une autre perspective à notre travail pourrait donc consister à effectuer des doubles marquages NeuN-BDNF, Iba1-BDNF et GFAP-BDNF, en vue d'identifier le/les phénotypes cellulaires impliqués dans l'autoinduction du BDNF, et, sans doute, dans l'effet allodynique/hyperalgésique de longue durée d'une seule injection de ce facteur neurotrophique au niveau spinal.

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